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## **Fibrous clays based bionanocomposites**

**Eduardo Ruiz-Hitzky<sup>\*1</sup>, Margarita Darder<sup>1</sup>, Francisco M. Fernandes<sup>1,2,3,4</sup>,**

**Bernd Wicklein<sup>1,5</sup>, Ana C.S. Alcântara<sup>1</sup>, Pilar Aranda<sup>1</sup>**

<sup>1</sup>*Instituto de Ciencia de Materiales de Madrid (ICMM-CSIC), c/Sor Juana Inés de la Cruz 3, 28049 Madrid (Spain)*

<sup>2</sup>*UPMC Université de Paris 06, UMR 7574, Chimie de la Matière Condensée de Paris*

<sup>3</sup>*CNRS, UMR 7574, Chimie de la Matière Condensée de Paris*

<sup>4</sup>*Collège de France, de la Matière Condensée de Paris, 11 place Marcelin Berthelot, 75231 Paris Cedex 05, France*

<sup>5</sup>*Present address: Department of Materials and Environmental Chemistry, Stockholm University, 106 91 Stockholm (Sweden)*

*\*Author for correspondence*

*E-mail: [eduardo@icmm.csic.es](mailto:eduardo@icmm.csic.es)*

## **Abstract**

Sepiolite and palygorskite are natural microfibrinous clay minerals whose particular structural, morphological and textural features are useful for the preparation of a wide variety of advanced nanostructured materials, essentially regarding their ability to render nanocomposite materials. The silanol groups located on the external surface of these silicates and their surface electrical charge are the centres for interactions with biopolymers, such as polysaccharides, proteins, lipids and nucleic acids. In the present review we introduce recent results from sepiolite- and palygorskite-based bionanocomposites showing the interest of these silicates compared to lamellar clays for diverse applications such as bioplastics and membranes, uses in biomedicine as drug delivery systems and adjuvants of vaccines as well as in tissue engineering. Other applications here discussed focus on environmental purposes, the use of bionanocomposites as components of sensor devices and bioreactors and as source of supported graphene.

## **Keywords**

sepiolite, palygorskite, fibrous clays, bionanocomposites, polysaccharides, chitosan, starch, phospholipids, gelatin

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## Nomenclature

AfB1	aflatoxin B1
CEC	cation exchange capacity
CMC	carboxymethyl cellulose
CO <sub>x</sub>	cholesterol oxidase
CTS-g-PAA/APT/SA	chitosan-g-PAA/palygorskite/alginate
DDS	drug delivery systems
DNA	deoxyribonucleic acid
DS	diclofenac sodium
HA	hydroxyapatite
LDH	layered double hydroxides
ML-PC	phosphatidylcholine monolayer
OGal	n-octyl- $\beta$ -D-galactoside
PAA	polyacrylic acid
PAAm	polyacrylamide
PC	phosphatidylcholine
PCL	polycaprolactone
PEG	polyethylene glycol
PHB	poly3-hydroxybutyrate
Sep-BL-PC/URE	urease immobilized on sepiolite with a phosphatidylcholine bilayer
Sep-ML-PC	phosphatidylcholine monolayer on sepiolite
Sep-ML-PC/URE	urease immobilized on sepiolite with a phosphatidylcholine monolayer
PLA	polylactic acid or polylactide

PVA	polyvinyl alcohol
SBSs	nonionic sugar-based surfactants
Sep/URE	urease on pristine sepiolite
TEM	transmission electron microscopy
URE	urease
WG	wheat gluten
WVP	water vapor permeability

## 1. Introduction and general concepts

After our earliest works reporting the assembling of biopolymers such as chitosan and other polysaccharides to smectite clays and layered double hydroxides (LDH) [1,2], we introduced in 2008 the concept of bionanocomposites in the Kirk-Othmer Encyclopedia [3]. Nowadays, the term bionanocomposites –equivalent to nanobiocomposites used by certain authors– is widely used to describe biohybrid materials composed of species of natural origin, mainly biopolymers, and inorganic solids of diverse structure and morphology assembled at the nanometric scale. These materials attract many scientists and engineers due to their extensive incidence in different application fields from environmental remediation to biomedical uses. Of particular relevance are bionanocomposites in which the inorganic component is a silicate belonging to the clay minerals family. Natural and synthetic smectites such as montmorillonite and Laponite<sup>®</sup>, respectively, are layered 2:1 charged silicates that are most largely employed to prepare clay-based nanocomposites. Recently, the involvement of clays showing non-lamellar structural arrangement such as tubular halloysite and imogolite, but more frequently sepiolite and palygorskite fibrous clays, become attractive as alternative nanofillers with an increasing use in the preparation of new bionanocomposites. The sepiolite and palygorskite fibrous clays offer relevant properties afforded by their unique morphological and surface features that give rise to the enhancement of mechanical properties associated with the fiber reinforcement, and also favoring their assembly to the biopolymer matrix by hydrogen bonding through the external silanol groups ( $\equiv \text{Si-OH}$ ) on the clay surfaces.

The crystalline structure of both sepiolite and palygorskite microfibrillar clays is related to talc-like ribbons parallel to the fiber axis that can be regarded as structural blocks alternating with structural cavities, named *tunnels*, associated with the internal

surface of the silicates that grow up in the fiber direction (c-axis) (Fig. 1) [4-6]. The tunnels acceding to the external surfaces of fibrous clays are termed as *channels*.

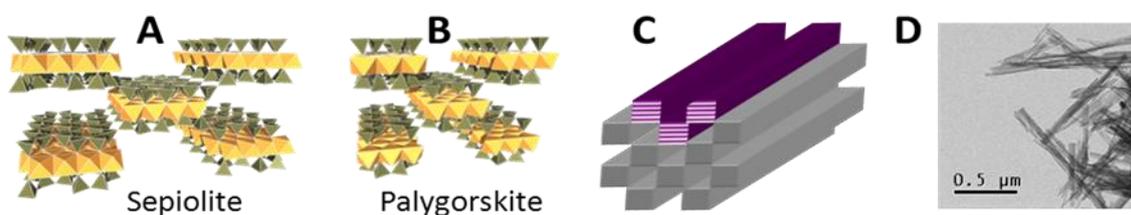


Fig. 1. Schematic representation of structural and textural features of fibrous clay minerals: A) sepiolite structure; B) palygorskite structure; C) ideal representation of the cross-section of sepiolite fiber based on Ruiz-Hitzky, 2001 [7]; D) TEM image of sepiolite (Pangel S9, from TOLSA commercial sample corresponding to deposits close to Madrid, Spain).

The ideal unit cell formula of sepiolite ( $\text{Si}_{12}\text{O}_{30}\text{Mg}_8(\text{OH})_4(\text{H}_2\text{O})_4 \cdot 8\text{H}_2\text{O}$ ) corresponds to a hydrated magnesium silicate, in which each structural block is composed by two tetrahedral silica sheets sandwiching to a central sheet of magnesium oxide-hydroxide. The periodic inversion of the tetrahedra silicon provokes the discontinuity of the silica sheets determining the presence of  $\equiv \text{Si-OH}$  on the "external surface" of the silicate fibers. In this way, silanol groups are directly accessible to diverse organic species including coupling agents and polymers allowing the preparation of nanostructured organo-inorganic materials [7-11]. Palygorskite, also known as attapulgite, shows an alike morphology than sepiolite but contains a higher content in aluminium replacing magnesium in the octahedral layers. Dimensions of the cross-section of sepiolite tunnels are  $1.06 \times 0.37 \text{ nm}^2$  whereas in palygorskite are  $0.64 \times 0.37 \text{ nm}^2$ , allowing the access of only small-size molecules such as water and methanol [7].

Composition and morphology of these fibrous clays can fluctuate very much from one deposit to another. Sepiolite shows typical fiber length of about 1-2 micrometer, although in certain cases (e.g. China, Finland) the fibers are much longer. Typical dimensions of the section of a sepiolite fiber from Vallecas (Spain) is 25 x 4 nm, which corresponds to about nine unit cells in width and only three in thickness [12]. In general, palygorskite fibers are longer with average size < 5 micrometers although in palygorskites of hydrothermal origin, it is frequent to find fibers with lengths over 20 micrometers [13].

Two types of water molecules are located inside the tunnels and the channels of both sepiolite and palygorskite silicates: i) *coordinated water* molecules, bonded to Mg<sup>2+</sup> at the edge of octahedral sheets, and ii) *zeolitic water* molecules, filling the intracrystalline cavities. The coordinated water is strongly bonded to the silicate making thermal treatments above 400 °C under dynamic vacuum necessary for its removal. Zeolitic water molecules are easily removed just by exposure to vacuum or by thermal treatment at about 100 °C. The loss of the coordinated water produces structural collapse of the structure (folding) and the fall down of the tunnels as revealed by XRD patterns and other techniques [14].

As indicated above, the surface properties of these fibrous silicates are the reason of their special ability to interact with many different compounds, including polymers, forming nanostructured materials and nanocomposites with implication in many diverse uses and industrial applications [11]. Layered clay minerals such as montmorillonite, are the most frequent clay nanofillers employed for the preparation of polymer-clay nanocomposites, in which the silicates are expected to be exfoliated (delaminated). In contrast, nanocomposites based on fibrous clays cannot suffer delamination processes because that would imply destruction of the silicate structure. Table 1 shows the most

relevant differences between sepiolite and montmorillonite, as typical fibrous and layered clays, respectively. Therefore, sepiolite and palygorskite based nanocomposites usually show disaggregation of the fibers within the polymeric matrix. To reach this goal it is recommended to employ starting clays of rheological grade in order to favor maximum clay defibrillation and so to maximize fibers dispersion. One of the advantages of fibrous clays compared to layered silicates (smectites and vermiculites) is the very high density of silanol groups, *ca.* 2 Si-OH groups/nm<sup>2</sup>, which allows hydrogen bonding in addition to van der Waals interactions at the polymer-silicate interface. Surface treatments of fibrous clays with organic surfactants were found to enhance their compatibility with low-polar polymers [15]. Organic derivatives of clays are formed by grafting organic groups onto the silicate surfaces through covalent bonds using silanes and other coupling agents [7,16,17]. In this way, it is possible to reach excellent adhesion/compatibility with many polymeric matrices that together with the strong anisotropy of these minerals results in a significant reinforcing effect on polymers, with the concomitant increase of the mechanical properties [11]. The single sepiolite fiber has a Young modulus of about 50 GPa, according to Fernandes [18].

Table 1

Main differences between a typical layered clay (montmorillonite) and a fibrous clay (sepiolite).

<b>Montmorillonite</b>	<b>Sepiolite</b>
Layered clay	Needle-like clay
1D nanoparticle	2D nanoparticle
High charge density	Low charge density
High Cation Exchange Capacity	Low Cation Exchange Capacity
Low density silanol groups (at the edges of the platelet particles)	High density silanol groups (covering all the external fiber surface)
High internal surface area	High internal & external surface area
Particles in layer stacks	Particles in bundles
Swelling clay able to delaminate	Non-swelling clay & non-exfoliable clay

Biopolymers are the most employed species from biological origin to prepare fibrous clay-based bionanohybrid materials. In this context, proteins, such as collagen and its gelatin derivative received special interest for the first preparations of sepiolite-bionanocomposites focusing on biomedical applications [19-21]. It is opportune to remember here that nanocomposites formed by a combination of hydroxyapatite (HA) with collagen or gelatine [22-34] are of particular interest due to the essential role of HA as the main mineral phase component of natural bone, resulting in bionanocomposite materials of interest for tissue engineering (artificial bone and bone repair) [21]. In fact, both structural proteins can be considered as an adhesive and biocompatible binder that could accelerate the recovery of damaged soft tissue [21]. Another interesting protein is zein, the major storage protein of corn, containing a large number of non-polar amino acid residues, which is used as a component of adhesives, food products as well as biodegradable plastics and as a drug-delivery matrix alone or as bionanocomposite-based systems [35-38]. Zein shows strong hydrophobicity, which renders it insoluble in water but soluble in hydroalcoholic media, hence allowing its assembly to inorganic components including fibrous clays, and eventually giving rise to functional bionanocomposites [37,38].

Polysaccharides, such as cellulose, starch and chitosan are the most abundant natural polymers on Earth [39]. They have been largely used in the development of biodegradable composite materials (*green nanocomposites*) providing benefits due to their low cost and positive ecological impact [3,40]. Cellulose and starch have been assembled to layered silicates, e.g., montmorillonite, but their low solubility in water and other polar solvents limit their use, making their combination to modified clays (organoclays) necessary. Alternatively, these polysaccharides can be transformed into

derivatives for their assembly to inorganic substrates [41]. As it will be discussed below, these polysaccharides show high tendency to assembly to sepiolite and palygorskite by hydrogen bonding and water bridge interactions between hydroxyls from both components. Polysaccharides such as alginate, pectin, xanthan, carrageenans, guar and locust bean gums, etc., provided of negatively charged groups, mainly carboxylate and sulphate, can also be assembled to fibrous clays through interactions between OH groups from both components. In this case, residual anionic functions can be further profited from for assembly of suitable positively charged compounds. Chitosan is a positively charged polysaccharide that in addition to this type of interactions can also be assembled to sepiolite and palygorskite through ionic bonding, because it may act as a polyelectrolyte that compensates the electrical charge of those inorganic components.

Other polymers of natural origin that are also used in the preparation of bionanocomposites are certain polyesters produced by microorganisms (*biopolyesters*): poly(lactic acid) or polylactide (PLA) and poly(3-hydroxybutyrate) (PHB). The most widely used polyester to prepare biopolymer–clay nanocomposites is PLA, which has been combined with modified smectites [3] and more recently assembled to sepiolite [42-44].

**Table 2** collects the most representative examples of biopolymers (Fig. 2) used in the preparation of bionanocomposites based on fibrous clays. There is also reference to incipient research to sepiolite-based hybrids containing biomolecules provided of latent bioactivity such as nucleic acids. The table also includes examples based on lipids such as phosphatidylcholine (PC), which, even though not strictly being biopolymers, show a self-assembly capacity able to drive to the formation of nanocomposites by interaction with smectites and fibrous clay minerals. Interestingly, these last systems can be

regarded as biomimetic interfaces, which are of utmost concern to an increasing scientific community [45].

Toxicity of sepiolite and palygorskite clay minerals is controversial as is apparently related to the fiber length and crystallinity, which are determined by the geological origin of these silicates [46]. From *in vivo* experiments it seems that fibers of lengths inferior to 5 micrometers do not cause mesotelioma in mice [46,47]. The effect of sepiolite and palygorskite on lysis of erythrocytes has been demonstrated by Oscarson et al. [48]. Sepiolite is considered nonhazardous according to European directives (e.g. 67/548/EEC) [49], being extensively used as additive in animal forage. *In vitro* and *in vivo* tests, as well as epidemiological studies, have also confirmed that at least sepiolite from Taxus Basin deposits in Spain does not constitute a health risk [50,51].

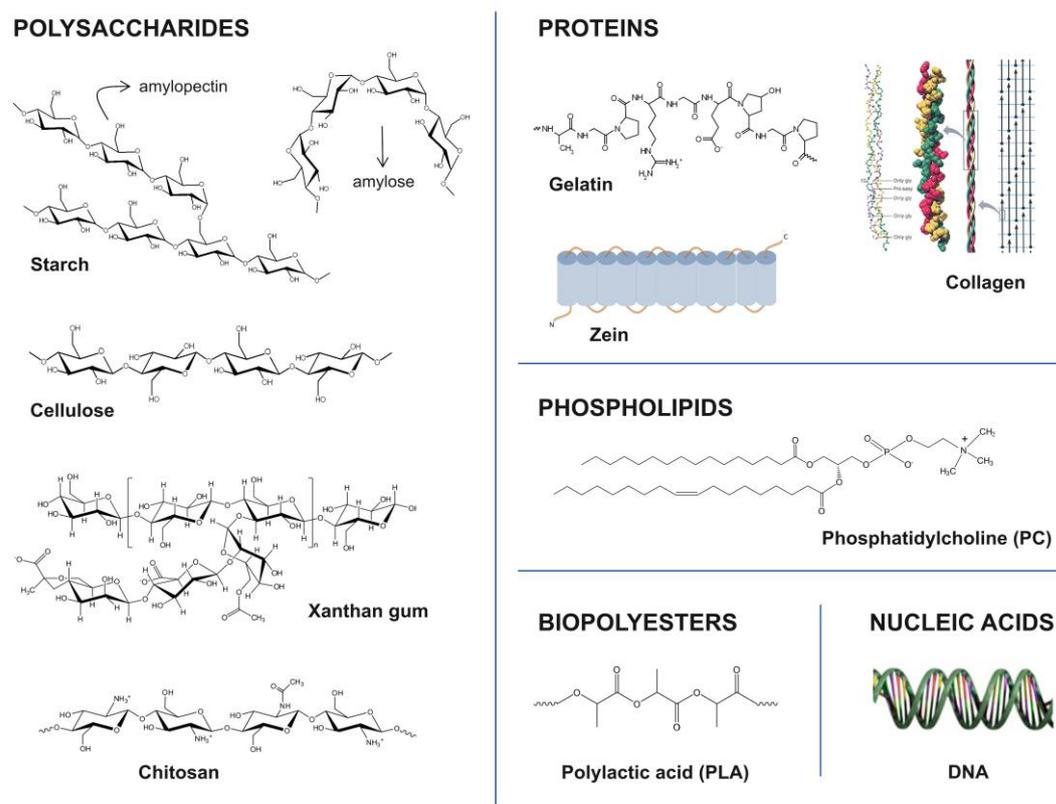


Fig. 2. Chemical structures or schematics of biopolymers commonly used in the preparation of bionanocomposites.

**Table 2**

Biopolymers used in the preparation of bionanocomposites based on sepiolite and palygorskite fibrous clays

<b>Biopolymers</b>	<b>Fibrous Clay</b>	<b>Properties/ applications</b>	<b>Authors and selected references</b>
Starch	Sepiolite	Bioplastics, improvement of mechanical properties	Chivrac et al., 2010 [52]
	Palygorskite	Foams: thermal & acoustical insulation, fire retardancy Superabsorbents	Ruiz-Hitzky et al., 2010 [53]; Darder et al., 2011 [54] Li et al., 2005 [55]
Cellulose (derivatives)	Sepiolite	Rheological additives	Chang et al., 1991 [56]
	Palygorskite	Superabsorbents Humidity control	Liu et al., 2010 [57] Yang et al., 2011 [58]
Chitosan	Sepiolite	Improvement of mechanical properties	Darder et al., 2006 [59]
	Palygorskite	Chemical sensors Superabsorbents; Drug delivery Environmental remediation	Darder et al., 2012 [60] Wang et al., 2011 [61] Pan et al., 2006 [62]; Cohen and Joseph, 2009 [63]
Xanthan	Sepiolite	Virus immobilization/ vaccine adjuvants	Ruiz-Hitzky et al., 2009 [64]
Biopolyesters (PLA and PCL)	Sepiolite	Bioplastics	Fukushima et al., 2009 [42]
			Fukushima et al., 2010 [43] Fukushima et al., 2011 [65] Hapuarachchi and Peijs, 2010 [44]
Collagen	Sepiolite	Tissue engineering	Pérez-Castells et al., 1987 [19]
			Olmo et al., 1996 [20] Herrera et al., 1995 [66]
Gelatin	Sepiolite	Improvement of mechanical properties	Fernandes et al., 2011 [67]
			Frydrych et al., 2011 [68]
Zein	Sepiolite	Bioplastics additive	Alcântara et al., 2008 [69]
	Palygorskite		Ruiz-Hitzky et al., 2010 [70] Alcântara et al., 2011 [38] [71]
Phosphatidylcholine	Sepiolite	Biomimetic interfaces Micotoxins sequestration	Wicklein et al., (2010) [72]
			Wicklein et al., (2011) [73] Wicklein (2011) [74]
DNA	Sepiolite	Supported DNA	Darder et al. (2010) [75]

## **2. Sample preparation and processing**

The basic assembly processes for the preparation of fibrous-clay based bionanocomposites regard mostly adsorption and occasionally covalent grafting [52,54, 59,67,72] of biopolymers or other biomacromolecules on the external clay surface. In general, these processes are highly suitable for the initial assembly of the two components but are not especially suited for the preparation of final materials. In fact, this assembly process can be viewed as a preliminary step allowing subsequent conformation using complementary techniques as mentioned in the following subsections.

### *2.1. Foams*

Applications like insulation, cushioning, packaging, or scaffolds for biomedical purposes usually require the processing of materials as cellular solids or low density foams [76]. In the search of ecological alternatives for insulation and packaging applications or of biocompatible implants for regenerative medicine, the current research is focusing on cellular solids based on naturally occurring and synthetic biodegradable polymers, including both polysaccharides and structural proteins (chitosan, starch or collagen) or polymers like PLA or polycaprolactone (PCL). Their combination with suitable inorganic solids may help to improve the mechanical resistance of the resulting bionanocomposite foams in comparison to the pristine polymer foams, and it may also afford other properties such as fire resistance [54]. Although the first attempt to prepare clay-reinforced hydrocolloid foams as ecological substitutes of commercially available polystyrene foams involved the use of smectites [77,78], recent works have shown the possibility of using fibrous silicates in

combination with polysaccharides and structural proteins for the development of rigid foams with enhanced toughness and low relative density [53,54,68,70].

The common techniques applied to develop cellular materials include solvent casting and particulate leaching, gas foaming, fiber knitting, or phase separation/emulsion freeze drying, amongst others [76]. But given that water is the solvent usually employed in the preparation of biopolymer-based nanocomposites, freeze-drying is one of the most suitable techniques to obtain bionanocomposite foams [79]. During the freezing step, the random growth of ice crystals creates the porous structure by pushing and accumulating the water-suspended components in the voids between adjacent ice crystals. Subsequently, sublimation under vacuum allows the removal of the ice template without collapse of the structure, resulting eventually in a low density porous material.

The morphology of the macroporous structures can be tuned by controlling the conditions of the freeze-drying process. For instance, aqueous colloidal suspensions can be unidirectionally frozen by placing the sample between cold fingers whose temperature is controlled by liquid nitrogen baths and ring heaters [79] or by dipping the sample container at a constant rate into a liquid nitrogen bath [80,81]. This novel procedure, which was denoted “ice segregation induced self-assembly (ISISA) process” [82], produces microchanneled structures well-aligned in the freezing direction. Alternative drying techniques that make use of supercritical fluids such as carbon dioxide [83], extrusion at controlled temperature [84], or foaming in a heated press using urea as blowing agent [85] have been also reported for the preparation of smectite-based bionanocomposites foams. Similarly, all these mentioned techniques could be extended to the processing of sepiolite-based analogues, whose development has been based uniquely on freeze-drying until the present time.

## 2.2. Films

In the field of advanced materials design, materials can be processed as films presenting highly desirable features for applications in packaging [86], membranes [87], coatings [88] and catalysis [89], or even in innovative applications such as support of electronic devices for medical science [90]. Currently, innovative techniques have been used in the development of functional films prepared from a combination of inorganic solids, generally clay minerals, with organic compounds giving rise to the well-known organic–inorganic nanocomposites [91]. Although the majority of works on nanocomposite films reports the use of layered clays such as montmorillonite [92], saponite [93] or laponite [94,95], recent works have demonstrated the possibility of employing sepiolite and palygorskite fibrous clays as the inorganic counterpart in the processing of hybrid films, providing the resulting material with improved mechanical and barrier properties not only in comparison to the polymer alone, but also when compared with smectite-based analogous materials [52,59,70,96,97].

Among the most diverse film processing strategies reported for hybrid materials [98], the following methods can be highlighted: i) solvent casting, which consists in the film formation from the casting of a polymer-clay mixture onto a support; after drying by evaporation of the solvent, the resulting films can be stripped off the support [99]; ii) spin-coating, where the spread of the clay or clay-polymer suspension over the substrate is carried out firstly by spinning at a low rotation speed, subsequently, at higher rotational speed to deposit oriented clay particles on the support plate by centrifugal force and to control the final thickness of the hybrid film [98]; and iii) Layer-by-Layer (LbL) assembly, which is a technique based on the alternate deposition of layers of oppositely charged polyelectrolytes or charged nanosized clay particles, suggesting that

electrostatic or hydrogen bonding interactions is the main driving force for the construction of ultrathin hybrid films [100]. In addition to dip-coating, which is usually employed to prepare the alternate layers, spin-coating has been also proposed as a faster method to produce self-standing films through LbL [101].

Within the scope of bionanocomposite films, including those based on sepiolite or palygorskite, solvent casting is the method mainly employed to form self-standing films during water evaporation and drying [99]. The majority of the involved biopolymers are highly hydrophilic, having water as the main solvent. Good distribution and a regular arrangement of the fibrous clays in the biopolymer matrix are the prime features of the bionanocomposite films produced by this easy and versatile method, where the high dispersion of the clay is the key for good properties of the final material [52,97]. Habitually, to achieve a proper dispersion of the fibrous silicate, it is necessary to keep a suspension of the clay under magnetic stirring [70] or sonication [97] during a long time, before and after mixing with the biopolymer. The spreading of the clay-loaded biopolymer should be carried out on a smooth surface, being frequently used plates of glass or Teflon, as they allow an easy removal of the bionanocomposite films after drying.

When the biopolymer is a thermoplastic polymer, such as PLA, PCL, wheat gluten (WG) and some starches, the processing of the bionanocomposite films based on fibrous clays is often carried out by two steps: firstly, a suspension of clay is mixed with the biopolymer by melting blend in an internal mixer. In this step, time, speed, and temperature applied in the mixing process can be varied in order to achieve a high dispersion of the clay in the polymeric matrix and increase compatibility between organic and inorganic components, obtaining better properties in the final product [42,52]. Usually, in the second step of the process, the films are obtained by

compression molding using a hot-plate press. The resulting films are achieved by the simultaneous application of heat and pressure, and in some cases the material obtained is characterized by forming homogeneous rigid films [102]. Thermo-pressed bionanocomposite films generally exhibit a morphology where sepiolite or palygorskite fibers are uniformly dispersed, randomly oriented and closely embedded in the biopolymer matrix, although some particles can remain agglomerated or bundled [103].

Briefly, although diverse methodologies can be applied in the processing of clay-loaded films, still few studies are reported for bionanocomposite films based on fibrous silicates, where solvent casting and hot-pressing are the techniques normally employed. Besides them, spin-coating and the LbL process, or even a combination of various of them, as demonstrated for other bionanocomposites [104], could be extended in the near future to the development of these new types of biohybrid films based on sepiolite and palygorskite.

### *2.3. Biomimetic interphases*

One way to obtain biomimetic interfaces is self-assembly of suitable molecules, which display certain structural and functional features that permit controlled assembly [105]. Such processes can occur by molecular self-assembly of dissolved molecules including supramolecular chemistry [105,106], self-assembly of preformed aggregates such as vesicles [107-109], or using Langmuir-Blodgett technologies [110,111]. In many of these processes, biological surfactants (biosurfactants) are frequently employed as they are especially appropriate for the design of biomimetic interfaces [112-114]. These biosurfactants display a similar polar/apolar structure as conventional surfactants, but with the additional quality of more sophisticated functional groups and structural features owing to their biological origin [115]. The hydrophilic headgroup can be

comprised of mono-, oligo-, or polysaccharides, peptides, or proteins. The hydrophobic tail may consist of saturated, unsaturated and hydroxylated fatty acids or fatty alcohols [115]. Naturally occurring biosurfactants are e.g. glycolipids, fatty acids, phospholipids, lipopeptides, and polymeric biosurfactants [116]. However, there is also an increasing contribution of synthetically produced biosurfactants like full peptide surfactants, sugar-based surfactants, or DNA surfactants [117-120]. Further advantages of biosurfactants besides the structural and functional variability are inherent biocompatibility, biodegradability, and non-hazardness [116].

### *2.3.1. Supported Lipid Membrane*

The potential of supported biomembranes has been explored by Wicklein et al. in assembling different lipid structures on the surface of sepiolite [72,73]. Lipid deposition was achieved either by liposome adsorption or by molecular assembly from organic media [74]. In both cases, the adsorption isotherms show the characteristic shape of a mono- and bilayer formation as function of the equilibrium phosphatidylcholine (PC) concentration. It is also revealed from this study that liposomal deposition is more effective for the formation of a lipid membrane in this specific system by requiring significantly lower equilibrium concentrations. The principal difference is that in aqueous media PC adsorbs as aggregates while in ethanol single PC molecules associate to the sepiolite. Liposome adsorption on solid interfaces can be described as a multistage process including i) first contact ii) liposome deformation iii) liposome rupture and iv) lateral lipid molecule diffusion for complete surface coverage [109,121]. This mechanism is believed to make bilayer deposition more efficient than molecular self-assembly.

On the other hand, in aqueous media, the ion-exchange capacity of sepiolite is likely

to dominate stronger during the first stage of PC adsorption as compared to adsorption from ethanol solution. The cation exchange capacity (CEC) of sepiolite is 15 mequiv/100g [7] which exactly matches the measured 15 mmol of adsorbed PC per 100 g sepiolite at the first isotherm plateau. This can be an indication that the initial stage of liposome adsorption is governed by a cation exchange mechanism. As for possible interaction mechanism, IR and NMR spectroscopic investigations strongly suggest hydrogen bonding both of the PC ester and phosphatidyl groups with the silanol groups of the sepiolite fibers [72].

Adsorption of phospholipids on sepiolite also leads to a change in surface hydrophilicity of the hybrid material. This can be examined by contact angle measurements of cast sepiolite-PC films (Fig. 3) revealing increasing contact angle values until the first plateau of the adsorption isotherm, whereas during the subsequent slope the contact angle decreases. The rise of the water contact angle can be related to increased hydrophobicity of the sepiolite fibers. This is the case for the lipid monolayer coverage with the hydrocarbon chains oriented bottom-up. With beginning bilayer formation gradually more polar headgroups are exposed at the air interface of the upper membrane leaflet and hence, the hydrophilicity increases.

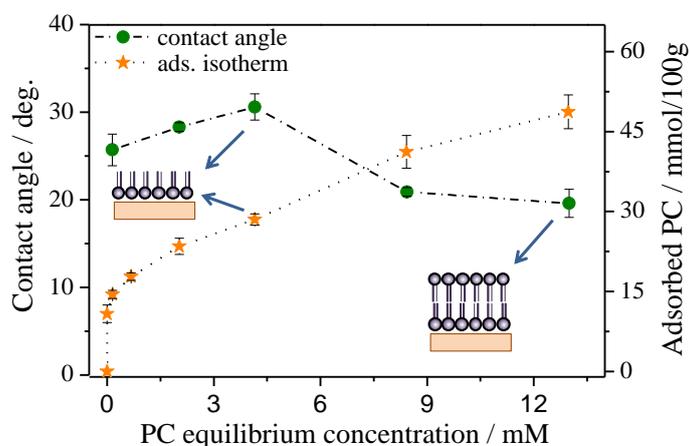


Fig. 3. Water contact angles on cast Sep-PC films are plotted together with the corresponding adsorption isotherm of PC on sepiolite obtained in ethanol.

Adapted from references [45, 72].

### 2.3.2. *Hybrid Bilayers*

Self-assembly also allows for the preparation of mixed lipid membranes that can be considered as a further development of the artificial lipid bilayer [122-125]. These hybrid layers are constituted of individual membrane leaflets, each consisting of different compounds. This strategy gives rise to a great variety of lipid-based membranes with diverse functionalities that can be important in different technological applications [123]. Nonionic sugar-based surfactants (SBSs), for instance, are a type of biosurfactants which are worth to be investigated for incorporation into these hybrid layers, e.g. for their antifouling properties [126,127] or protein stabilization ability [128-130].

Such a hybrid interface has been assembled on sepiolite, composed of an inner phosphatidylcholine monolayer (ML-PC) and an outer leaflet of n-octyl- $\beta$ -D-galactoside (OGal) by subsequent monolayer deposition from the corresponding biosurfactant solutions [73]. Incubation in OGal solutions of increasing concentration gives the OGal adsorption isotherm that follows a sigmoidal shape. This is being typical for adsorbates that show intermolecular cooperativity based on van der Waals interaction between hydrophobic moieties [131]. In particular, nonionic surfactants have been observed to adsorb at hydrophobic solid/water interfaces in this manner [132]. As for a growth mechanism, it is assumed that the PC monolayer serves as anchor and nucleation site for the growth of the OGal outer membrane leaflet by hydrophobic interaction between the hydrocarbon chains [133]. This assumption is also supported by Persson and co-workers who investigated adsorption of n-dodecyl- $\beta$ -D-maltopyranoside

on hydrophobized surfaces based on silane and concluded insertion of alkyl chains of the sugar-based surfactant into the hydrophobic silane layer as anchoring mechanism [134].

The saturation level of OGal on the PC monolayer is calculated from the adsorption isotherm (127 mmol/100g), which corresponds to a molecular packing density of 4.7 OGal molecules per nm<sup>2</sup>. This is about two times the monolayer packing density of n-octyl- $\beta$ -D-glucoside at the air/water interface [135]. The excess of OGal might be explained in terms of hydrophobic aggregation or hemimicelle formation [136-137]. This is a phenomenon observed for both ionic and nonionic surfactants on hydrophobized solids [138] and should therefore also be noted on Sep-ML-PC due to the hydrophobic character of its surface. In consequence, this mechanism can lead to increased molecule packing density and might explain in the present case the elevated packing density of OGal on Sep-ML-PC.

The adsorption of OGal molecules causes an alteration of the surface hydrophilicity of the resultant material as evidenced from water adsorption isotherms [73]. Using Park's model for isotherm analysis [139] reveals that pristine sepiolite fibers are very hydrophilic while the PC monolayer makes the surface more hydrophobic. However, with increasing OGal adsorption at the ML-PC surface water sorption is gradually enhanced due to the elevated number of galactoside headgroups at the solid/liquid interface.

### *2.3.3. Other types of supported membranes*

In addition to biosurfactant-based coatings, recent works have reported the interest in mimicking other types of membranes involving proteins and polysaccharides. Among them, a recent example describes the preparation of supports coated with a layer of the

glycosylated protein mucin, which may resemble a mucous membrane and interact actively with host proteins adsorbed on this coating [140].

Similarly, the assembling of polysaccharides to solid supports including clay minerals can also offer a biocompatible interface to accommodate biological elements and preserve their bioactivity. Thus, the surface of sepiolite fibers was modified with the anionic polysaccharide xanthan gum with the aim to develop a biointerface that mimics features of mucous membranes in their interaction with virus particles [64]. Xanthan gum consists of a  $\beta$ -(1 $\rightarrow$ 4)-D-glucopyranose glucan backbone with side chains of  $\alpha$ -(3 $\rightarrow$ 1)-linked D-mannopyranose-(2 $\rightarrow$ 1)- $\beta$ -D-glucuronic acid-(4 $\rightarrow$ 1)- $\beta$ -D-mannopyranose on alternating residues, being about 40% of the terminal mannose residues 4,6-pyruvated [141]. Modification of the sepiolite fibers takes place by spontaneous assemblage of xanthan, driven by hydrogen bonding interactions between the hydroxyl groups of the polysaccharide and the silanol groups located at the external surface of the silicate. The biohybrid material presents water retention ability and provides a favorable environment for biological species. It also bears the negative charges coming from xanthan, which can electrostatically interact with the positively charged surface of the influenza viral particles. Thus, the resulting biointerface would mimic the interaction of natural mucosa with virions during infection processes.

### **3. Bioplastics and membranes**

Currently, the major part of used plastics are derived from non-renewable petroleum and have a relatively short period of usage before being discarded, such as disposable utensils, diapers, trash bags, agricultural films, food packaging, and medical devices. In

this sense, the interest in using renewable biomass for the manufacture of high-quality, cost-competitive, and biodegradable consumer goods has been increasing in the recent years as a means to reduce the dependence and consumption of petrochemical feedstock and to diminish environmental pollution [142-143]. Packaging films, containers, and coatings made of natural polymers such as polysaccharides (cellulose derivatives, starch, alginate...) and proteins (collagen, gelatin, soybean protein, casein, and zein) are receiving considerable attention due to their low cost, high biocompatibility, and biodegradability, giving rise to the so-called bio-packaging materials or bioplastics [40,144,145]. Thus, bioplastic materials can be characterized as materials derived from renewable biomass sources, in which at least one step in the degradation process is through metabolism in the presence of naturally occurring organisms [86,146]. Together with the polymers found in nature, the group of biopolymers also includes those polymers produced by classical chemical synthesis using renewable bio-based monomers or mixed sources of biomass and petroleum (i.e. PLA and bio-polyesters) as well as polymers produced by micro-organisms or genetically modified bacteria (polyhydroxybutyrate, xanthan, curdlan or pullan) [147]. Most of these polymeric matrices have the ability to form films or to be molded into objects [148].

Unfortunately, the applications of bio-based plastics and films have several important limitations, which are caused by their relatively poor barrier and mechanical properties [149-151]. Therefore, natural or synthetic clay minerals, especially layered silicates such as montmorillonite and organically modified montmorillonites, are additives usually incorporated to enhance such properties of the biopolymer matrix, originating in this case bionanocomposite materials that can be applied as reinforced green plastics [3,40,152,153]. Owing to nanometer-size clay particles dispersed within the biopolymer matrix, many of these bionanocomposites exhibit improved physicochemical properties

when compared with the pure polymer, usually without the need for additional and cost-increasing processing or post-treatment procedures. For application in food packaging and due to the biodegradability of the involved biopolymers, these bionanocomposites are not only suitable for food protection, but can also be considered a more environmentally friendly solution because after final degradation only inorganic, natural minerals will be left over [154]. In this way, they help to reduce the use of petroleum-derived plastics as packaging materials [148].

The use of fibrous clays, such as sepiolite or palygorskite, as nanofillers into bio-based matrices is less studied than layered clays, but it becomes more and more attractive as an alternative to smectites due to various interesting characteristics: the large aspect ratio of these 2-nanodimensional particles, the presence of OH groups at their external surface and the possibilities of diverse functionalization to make them more compatible with the polymer matrix [11]. In this way, compared to conventional bionanocomposites films based on layered clays, sepiolite and palygorskite can show a higher reinforcement effect and good dispersion, as a result of the high interactions between the biopolymer matrix and the fibrous clay nanofiller. This strong affinity was confirmed by Darder et al. [59], studying the assembly of the positively charged polysaccharide chitosan to sepiolite. In this work, the resulting bionanocomposites were processed as self-supported films, in which the sepiolite fibrils seemed to be very well integrated into the biopolymer matrix, producing an increase in the elasticity modulus about 3 times higher than that of the pristine polysaccharide film. In addition, these chitosan-sepiolite bionanocomposites films have also been tested as membranes for N<sub>2</sub> separation, being a promising material in membranes process [155].

Besides chitosan, our group has extended the preparation of water soluble biopolymers/microfibrous clays bionanocomposites involving sepiolite and palygorskite

to other polysaccharides: alginate, pectin, starch, xanthan, carrageenan, carboxymethylcellulose (CMC) and hydroxypropylmethylcellulose (HPMC) [156]. The resulting bionanocomposites not only exhibit improved mechanical properties, but also a significant barrier to ultraviolet light and to the passage of water, reducing the water vapor permeability (WVP), which make them very attractive for use in food packaging or coatings. Moreover, these films also offer interesting results for the retention of heavy metals such as copper, chromium and lead, which together with the biocompatibility and biodegradability afforded by the biopolymer may enlarge the scope of applications as bioplastics, for instance in the agricultural sector.

Strong interactions at the nanometer range were also observed between the structural protein gelatin and sepiolite in the development of transparent and homogeneous bionanocomposites films [21,67,97]. One of the most salient features attributed to the interactions between both components in sepiolite-reinforced gelatin materials was the improvement of mechanical properties, reaching a 250% increase in the elastic modulus at 50% (w/w) clay loading. Nevertheless, these studies demonstrated that sepiolite reinforcement is more effective at low clay loading, suggesting that specific clay-protein interactions play a significant role in obtaining structurally high efficient composites. The sepiolite fibers seem to have a great influence on the matrix crystallinity, which is most likely attributed to the fitting of the collagen-like triple helix of the renatured gelatin along the external surface channels of sepiolite [67].

Recently, sepiolite was also incorporated in bioactive packaging based on gelatin-egg white films containing clove essential oil [157]. In contrast to the improvements in barrier and mechanical properties observed in absence of the clove oil, its presence in sepiolite-reinforced films produced a decrease in Young modulus, tensile strength and water barrier properties, due to the plasticizing effect of the oil. In spite of that, the

complex system offers other interesting antioxidant and antimicrobial properties that seem to be originated by the presence of sepiolite fibers. They may partially hinder the protein-clove oil interaction and favor the controlled release of protective protein components and eugenol from the film matrix.

Wheat gluten (WG), a multicomponent agroproduct from the wet-milling of wheat flour, is also being used as component in packaging films or edible coating materials [158]. This biopolymer is comprised of storage proteins, which confer viscoelasticity, selective permeability, and biodegradability. A detailed study of the homogeneous and transparent thermo-pressed WG films using pristine palygorskite as nanofiller was reported by Yuan et al. [103]. Palygorskite particles demonstrated a good dispersion and high compatibility with the biopolymer matrix, showing the best results of viscoelasticity for 7% (w/w) of clay loading. In addition, the increase of the biodegradability also was observed, with disaggregation and dwindling of WG/palygorskite films after 15 days of burial (Fig. 4a).

Within the perspective of biodegradable polymers for bioplastic applications, PLA or PCL are very interesting because of their good film-forming ability and flexibility. Liu and co-workers [159,160] have prepared nanocomposites based on PCL and palygorskite by solution mixing trying to understand the rheological characteristics of this system. The presence of palygorskite serves as nucleation sites for crystallization of PCL. More complex procedures implying controlled ring-opening polymerization and click chemistry has been applied to the preparation of PCL/palygorskite bionanocomposites using a previously functionalized palygorskite by treatment with 3-chloropropyltrimethoxysilane [161]. Methodologies that implies the treatment of sepiolite with organosilanes has been applied to the preparation of PCL/sepiolite

nanohybrids that are used for incorporation as nanofiller in a PCL matrix by melt-blending processing [162].

Fukushima et al. [42], have demonstrated the influence of adding sepiolite to both PCL and PLA biodegradable matrices. Easy dispersion without the need of organic modifiers or compatibilisers and high thermal-mechanical enhancements were obtained for these bionanocomposite plastics, probably due to strong polymer/filler interactions. Furthermore, the presence of sepiolite has an important effect on the biodegradation of PLA and PCL bioplastics in compost [43]. PCL undergoes a mechanism of surface degradation with less influence of the sepiolite fibers (Fig. 4b). In contrast, PLA follows a mechanism of bulk degradation, which is partially delayed by sepiolite, most likely due to a preventing effect of the fibers on polymer chain mobility and/or PLA/enzymes miscibility. The hydrolytic degradation of PLA in pH 7.0 phosphate buffer is also delayed by sepiolite (Fig. 4c) [65], which could be attributed to its inducing PLA crystallization effect and/or to its high water uptake that leads to the reduction in the amount of water available for polymer matrix hydrolysis. However, at 58°C the presence of sepiolite filler does not significantly affect the degradation trend of PLA, achieving similar molecular weight decreases for the bionanocomposite material and pristine polymer film (Fig. 4d). This could be due to an easy water region molecules access to the amorphous and semi-crystalline regions, initiating fast hydrolytic chain scission in both materials.

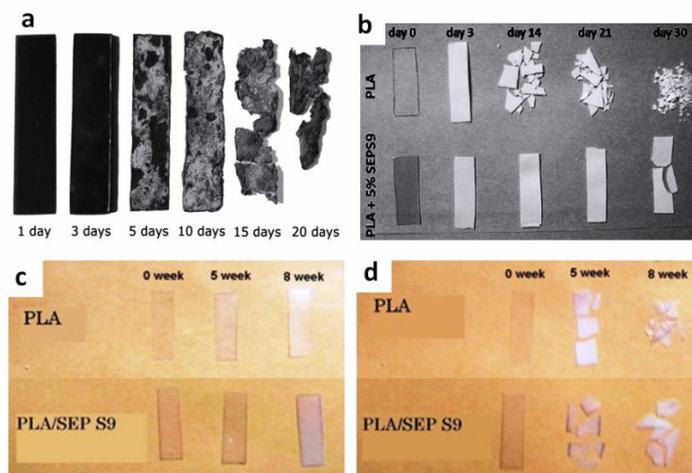


Fig. 4. Change of appearance during the biodegradation of WG/palygorskite (a) and PLA/sepiolite (b) in compost, as well as during the hydrolytic degradation of PLA/sepiolite at 37°C (c) and 58°C (d). (Image (a) reprinted from [103]; Copyright 2010 Elsevier. Image (b) reprinted from [43]; Copyright 2010 Elsevier. Images (c,d) reprinted from [65]; Copyright 2011 Elsevier).

Given that the low water resistance is an important disadvantage in view to utilize bionanocomposites as packaging materials, especially in wet environmental conditions [149], the use of additives that may help to overcome this drawback is of special interest. In this way, Alcântara et al. [38] have reported the modification of sepiolite and palygorskite fibers with zein, a hydrophobic protein from corn, with the aim to produce bio-organoclays as a green alternative to conventional organoclays involving alkylammonium modifiers. Thus, zein-fibrous clay bionanocomposites seem promising as biofillers for different polymeric matrices. Interestingly, this novel type of biocompatible filler was tested in hydrophilic matrices, such as alginate polysaccharide films, helping to increase the stability in water of this type of biopolymer matrices, while producing a plasticizing effect. In addition, the materials that incorporate zein-based bio-organoclay can be promising for membrane applications in diverse processes, since they also

present better water vapor and gas barrier properties than pristine polysaccharide films (Fig. 5). This property can be attributed to the good distribution of the bio-organoclay particles within the biopolymer matrix, originating a tortuous path for diffusion of the molecules of water or gas. Thus, these biocompatible fillers can be a green alternative to currently employed synthetic fillers, mainly for food packaging applications. Analogously, the surface modification of sepiolite was the strategy employed by Chivrac et al. [52] to develop starch-sepiolite nanocomposites. In this case, sepiolite organically modified with cationic starch was used as filler in plasticized starch films. In comparison with pristine sepiolite and organo-montmorillonites, organo-sepiolite produced a higher increase of Young's modulus and stress at break of the reinforced starch films. Such behavior is due to the good affinity between the sepiolite biomodified fillers and the polysaccharide matrix, where the cationic starch acts as compatibilizer between both components.

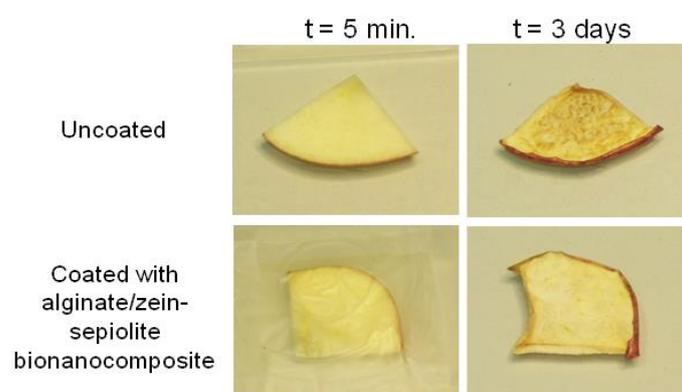


Fig. 5. Evaluation of the protective role of alginate films loaded with sepiolite modified with zein against oxidation of apple slices.

Taking into account that the addition of fibrous clays can optimize the barrier properties of a system and that sepiolite has demonstrated potential usefulness as

membrane for gas separation either alone [163] or associated with polymers of different nature [38,155,164], the use of bionanocomposites materials based on fibrous clays, despite that it is still in its beginnings, is a promising alternative to develop gas separation membranes, opening new possibilities of research within this field.

#### **4. Biomedical applications**

Clay based biohybrids are increasingly used in biomedical applications, in fields such as drug delivery, vaccination, wound dressings, and tissue engineering. The reason for this trend is the recognized biocompatibility of these materials and inherent non-toxicity. Another important feature is the ease with which clays can be modified and biorelevant properties such as surface charge, adsorbed biomolecule density, or hydrophilicity can be precisely tailored.

##### *4.1. Drug delivery*

Controlled drug delivery is required to avoid overdosing or underdosing episodes during the treatment, allowing the release of constant levels of the pharmaceutically active agents within the therapeutic dose. The use of clay minerals as carrier of pharmaceuticals offers numerous advantages for this purpose such as their inherent biocompatibility, low cytotoxicity, and precise tailorability of surface properties [165-168], which differ them from other current delivery systems like polymeric or viral vectors [169,170]. In fact, clays as additives in pharmaceutical formulations have already a long history starting in the 1960s [166]. The traditional use of pharmaceutical-grade clays such as smectites (bentonites, saponite), kaolinite, or fibrous clays (sepiolite, palygorskite) is related to their rheological properties, allowing them to act as

emulsifiers and stabilizers of pharmaceutical formulations and also as suspending agent for hydrophobic drugs [171-173]. Another important effect of clays is the retarded release of surface adsorbed pharmaceuticals, helping to modulate the drug release profile [166]. Pristine clays have been shown to retain drugs mainly by adsorption mechanisms such as ion-exchange reactions or intercalation into the interlamellar space of smectites [165,167,174]. A cationic (basic) drug is loaded via ion-exchange onto a suitable clay particle and subsequent to administration into an organism unloaded by ion-exchange with biological cations, replacing the drug [175]. These adsorption processes could be effectively enhanced and controlled by clay surface modification with biopolymers, as for instance regarding the affinity of the clay for nonionic actives or for hydrophobic drugs, which currently represent a growing fraction of newly developed pharmaceuticals. Another critical issue for many pristine clays in drug delivery applications is their tendency to flocculate at high ionic strength such as in physiological media [176]. Enhanced colloidal stability under such conditions can be imparted by adsorbed polymers like for instance polyethylene glycol (PEG) on laponite [177]. These few examples already highlight the possibilities of biopolymers for modifying clay surface properties, carrier-drug interactions, drug release profiles, and site specificity of the drug conjugate.

Among hybrid and biohybrid materials, biopolymer-clay nanocomposites are promising carriers for drug delivery systems, as they offer interesting properties such as swelling, film forming ability, bioadhesion, and cell uptake [168]. On the other hand, clays as fillers in polymeric delivery systems have demonstrated their capacity for substantial improvement of the mechanical and rheological characteristics of these carriers [178]. Although most part of the research has usually focused on layered silicates, fibrous clay minerals are also an appropriate carrier for drug delivery, showing

a good, direct interaction with several drugs, like  $\beta$ -blockers and 5-aminosalicylic acid [166] for instance, or offering the possibility of previous (bio)organic modification. Thus, nanocomposites involving the assembly of sepiolite with the biocompatible polymer polyvinyl alcohol (PVA) were proposed for the controlled release of rifampicin [179]. Instead of acting as carriers, the role of the sepiolite fibers dispersed within the drug-loaded polymer is to reduce the swelling capacity and the water migration into the PVA matrix and thus, adjusting the release rate of the rifampicin.

Concerning the use of naturally occurring polymers in association with fibrous clays for this purpose, a recent work reports the preparation of chitosan-palygorskite bionanocomposites processed as microspheres by means of spray-drying [61]. These materials were evaluated as carriers for oral delivery of diclofenac sodium (DS) with controlled release properties. The presence of palygorskite is not only beneficial to increase the isoelectric point and to obtain uniform microspheres with a narrow size distribution, but it also avoids the release of DS in gastric fluid, allowing its complete and controlled delivery in the intestinal region.

Palygorskite has been also incorporated in more complex biohybrid systems involving sodium alginate and an additional polysaccharide, chitosan [180] or CMC [181], which is grafted to the synthetic polymer poly(acrylic acid) (PAA). In these cases, the superabsorbent properties of the bionanocomposites were combined with specific abilities of the incorporated alginate for conforming the system as beads, through ionic cross-linking by means of  $\text{Ca}^{2+}$  ions. Active species like the drug DS may be entrapped within the resulting network (Fig. 6), allowing the preparation of new drug delivery systems (DDS). These beads are pH sensitive allowing a controlled release by changing the pH and controlling the amount of attapulgite, which has an important effect on the swelling ability

of the system. In this way, these materials can avoid leaching of the loaded drug in gastric fluid conditions, while allow its controlled release in the intestinal tract.

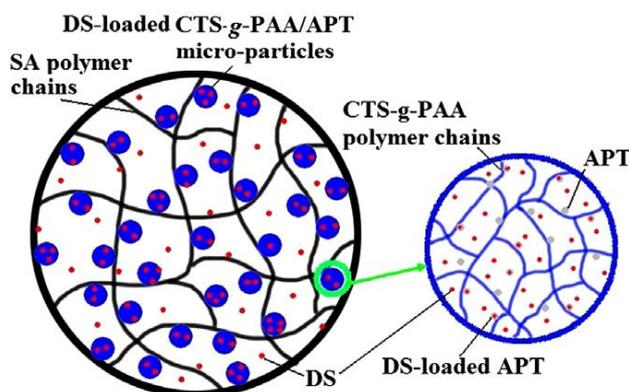


Fig. 6. Schematic structure of chitosan-g-PAA/palygorskite/alginate, noted as CTS-g-PAA/APT/SA, composite bead. (Reprinted with permission from [180]. Copyright 2009 Elsevier)

Bionanocomposite materials showing drug release properties are also of great interest for wound-dressing applications, as they may offer high water uptake and non-cytotoxicity together with other interesting features like mucoadhesivity. For instance, a recent example reports the successful evaluation of chitosan-montmorillonite nanocomposites for this purpose [182], showing biocompatibility and the ability to stimulate cell proliferation and wound healing. Based on this concept, bionanocomposites based on fibrous clays could be also considered as promising candidates for this kind of applications.

#### 4.2. DNA non-viral transfection

Pioneering works by Choy and colleagues show the ability of nucleic acids to assemble related clay materials such as magnesium-aluminium LDHs, following an ion-exchange mechanism [183-185], in which the LDH acts as a positively charged host able to intercalate negatively charged DNA. The arrangement of its chain conformation has

been determined from X-ray diffraction techniques [183-185] and investigated by Thyveetil et al. by computer simulation [186]. Interestingly, this biohybrid system allows the DNA transfer to the cell nucleus based on the shielding effect of the DNA's negative charge by the inorganic matrix through the cell membrane [165,184]. Contrarily to LDH solids, sepiolite and palygorskite exhibit negatively charged surfaces. However, in this case DNA can assemble to sepiolite fibers through hydrogen-bonding interactions with the external silanol groups present on this silicate. DNA chains of different length are spontaneously adsorbed from aqueous media on the external silicate surface following adsorption isotherms that show the high affinity of nucleic acids towards sepiolite [75]. Moreover, it has been recently observed the protection toward degradation of the sepiolite adsorbed DNA by the action of a DNase enzyme [75]. This type of systems is potentially of interest in view to apply fibrous clays as non-viral vectors for gene transfection. In fact, the transfer of exogenous plasmid DNA into bacterial cells using nanosize acicular solids has been recently investigated by Yoshida [187] showing the efficient possibility of controlled transformations of *E. coli* and other Gram-negative and Gram-positive bacteria using Spanish sepiolite with characteristic fibers inferior to 5  $\mu\text{m}$  of length [188].

#### *4.3. Vaccines*

Efficacious vaccines greatly rely on their stabilization on particulate carriers that can enable easy administration of immunogenic species, their protection from proteolysis, improved thermal storage performance, and afford an adjuvant effect of the solid vehicle [189]. To date, the great majority of particulate vaccine carriers are of soft matter, like liposomes, micelles, virosomes, polymeric nanoparticles, polymersomes, emulsions, and dendrimers [189,190]. However, inorganic, solid nanocarriers are

currently receiving increased attention due to their advantages like low cytotoxicity, controllable cargo release, particles-size control, and feasible functionalization [169]. Especially iron oxide nanoparticles and to a lower degree calcium phosphate and layered double hydroxide materials have found applications in cellular transfection for localized drug delivery [169]. However, the only approved inorganic antigen carrier at the moment is Alum, an aluminum hydroxide gel [191]. Even though it has been the standard adjuvant of choice for many decades, shortcomings like strong antigen-adjuvant interactions causing protein structure alteration and insufficient thermal stabilization are becoming evident [191]. Hence, new vaccine carriers are being sought and clays have been recognized as potential candidates due to their large protein adsorption capacity and inherent biocompatibility. Patil et al. [192] prepared a bentonite adjuvated vaccine against haemorrhagic septicaemia in calves which gave a longer-lasting immunity as compared to the alum supported vaccine. On the other hand, it has also been observed that adsorption of antigens on pristine clays can alter their protein structure which is manifested in decayed immunogenicity of the antigen and eventually, compromise the vaccination efficacy [64,193]. Therefore, Rytwo et al. [193] explored the ability of organically surface-modified montmorillonite to accommodate antigens related with the infectious bursal disease virus in the attempt to prepare efficacious subunit vaccines. In this study, it could be shown that the organic modification of the clay with berberine permitted the co-adsorption of two antigenic proteins, heat-labile enterotoxin and viral protein 2, under prevention of conformational protein alterations as evidenced from infrared spectroscopy while the secondary protein structure was changed on pristine clay. The retained bioactivity of these organo-clay associated proteins enabled efficacious binding to cellular GM1 receptors, an important step for stimulation of an immune response to the infectious bursal disease virus.

Another approach was undertaken by Ruiz-Hitzky et al. who used the biopolymer xanthan for surface modification of sepiolite to design an influenza vaccine [64]. Herein, the governing idea was to provide an environment for the adsorbed influenza virus similar to the nasal mucous, the natural location of influenza virus entry into an organism. The formation of stable suspensions of this bionanocomposite offers the possibility for nasal, needle-free delivery of the vaccine, a requirement often demanded for its ease and low costs [194]. Influenza A virions adsorbed on sepiolite-xanthan in a dispersed manner and in large quantities, driven by electrostatic attraction between the negatively charged carboxylate sites of the polysaccharide and the cationic centers on the virus surface. Conversely, virus immobilized on the pristine sepiolite surface formed agglomerates. Immunization of mice and posterior challenge with infectious influenza A virus demonstrated the high level of seroprotection imparted by this vaccine due the conservation of immunogenicity on sepiolite-xanthan. This has been attributed to the tissue like environment formed by the xanthan coating, possibly avoiding strong electrostatic interactions with highly polar surfaces that can drive to adsorption induced alterations of the secondary protein structure of antigens and subsequent diminishment of immunogenicity such as it is sometimes the case for standard aluminum containing adjuvants [191]. Another advantage of sepiolite based vaccines originates from its microfibrinous texture, which can contribute to elicit mucosal immune responses provoked from irritation of the nasal mucous by the fibers. This can help to improve the efficacy of vaccination and thus, reduce the necessary dose for immunization.

Another important issue in vaccination is the thermostability of vaccines, which becomes critical in stockpiling of pre-pandemic vaccines and in discontinuous cold-chains, e.g. in low-income countries [195]. But also stability against accidental freezing during transportation and storage can compromise vaccine efficacy (ca. 30% of all

vaccines are freeze-sensitive) [196]. Both scenarios represent economic and healthcare threats in the multi-million dollar range [196]. Therefore, most recently, an influenza A vaccine has been developed by the Ruiz-Hitzky's group based on sepiolite presenting a bilayer lipid membrane [74,197]. Functional studies with this vaccine revealed improved thermal stability at elevated temperatures until 48°C together with enhanced resistance against lyophilization induced antigen denaturation as often seen for Alum stabilized antigens [191,198]. This improvement in thermal stability is tentatively attributed to a twofold mechanism; a combination of thermal insulation and creation of a chemical microenvironment by the sepiolite-lipid biohybrid [197]. Related to their microporous texture, sepiolite fibers have a very low thermal conductivity (0.04 W/m k) [199,200] which makes an insulating effect imaginable. In addition, the biohybrid forms small, micron-sized agglomerates as observed from lyophilized suspensions. These agglomerates might form some kind of thermally protective scaffold for adsorbed influenza virions. The second part of the hypothesized mechanism is linked to the importance of a specific microenvironment. It is known for many adjuvants, such as for instance Alum, that they can create a chemical microenvironment with specific pH values, electrolytes, and ionic strength that can vary significantly from the bulk solution conditions [191,201]. Such microenvironments have been shown to influence the thermal stability of associated antigenic species [201]. Therefore, it might be reasonable to attribute similar effects to the sepiolite-lipid microenvironment.

#### *4.4. Tissue engineering and scaffolds*

Most of the tissue engineering applications in the field of bionanocomposites containing a mineral phase relates more or less directly to hard tissue replacement or healing. In this context, bone stands as the primary organ requiring surgical intervention

with around one million cases per year [202]. The socioeconomic implications linked to the development of efficient bone substitutes and scaffolds are thus of dramatic importance and stand as the driving force for the development of new, more efficient and less expensive materials.

Presently, the main strategies for the development of bone substitutes and scaffolds are the preparation of collagen-hydroxyapatite bionanocomposites that mimic the morphology and function of the natural material [203-205] or the replacement of the calcium phosphate moiety by silica [206-209]. Aside from the composition comprising collagen and hydroxyapatite, the development of performing materials for bone replacement is also dependent on the organizational state of collagen. The organization of the several hierarchical orders of collagen, from the triple helix to the supramolecular assembly of the collagen fibrils is another key issue to attain the desired mechanical properties.

The preparation of collagen based bionanocomposites using fibrous clays as inorganic moiety is incipient. Using mineral particle whose size is predefined a priori encloses some advantages which have not been fully explored in the tissue repair related bibliography. Composites based on sepiolite and collagen are amongst the more studied in the field of materials for tissue repair based on clays. The first work reporting the joint use of these two components described uniquely the adsorption of collagen in the outer channels of sepiolite [19]. Works by Gavilanes and co-workers [210,211] continued the same trend and reported the synthesis and application of materials that associate a collagenous matrix and sepiolite in 0.6:1 ratio respectively. The first application of such complexes was development of culture plates for fibroblast growth. After covering a culture plate with the protein-clay complex and seeding with human skin explants, the authors have observed fibroblast outgrowth, suggesting that the prepared hybrid

complex was biocompatible. Such promising results laid the basis for the progression of sepiolite-collagen complexes towards in vivo experiments. Herrera and co-workers [66], have implanted dried collagen-sepiolite complexes bearing the same protein to clay proportion in surgically created rat calvaria defect. Although the authors have not found the implants to induce bone formation they have reported full resorption and subsequent healing through osseous regeneration. Also important to refer is that there was no inflammatory response besides that induced by the surgical procedure. To some extent this work clears the doubts regarding the toxicity of fibrous particulate silicates, often associated with asbestosis [212]. On the other hand, the toxicity of mineral phases in biological composites is not at all a solved matter. Malafaya and Reis [213], for instance, have coined the term “calcium phosphate cytotoxicity” referring to the observed toxicity of a commonly accepted mineral for bioapplications.

## **5. Environmental applications**

Environmental applications of clay-organic based materials are known since the Antiquity and for instance, in Ancient Rome, clays and soil were combined with decaying urine to produce enhanced efficiency in laundry processes [214]. As clays are an abundant, low-cost and non-toxic natural resource they result especially attractive for environmental protection addressed to reduce hazardous species [215]. Hence, there is a growing field of research devoted to the study of more effective and environmental friendly modifications of clays, in particular for drinking water treatments [216]. In this context, the modification of clays with biopolymers results an attractive alternative for pollutants removal [214]. For instance, the possibility of combining adsorbent properties and ion-exchange capacity in

chitosan-clay bionanocomposites has been profited for recovery of azo dyes and heavy metal ions [60].

The assembly of biopolymers to sepiolite and palygorskite clay minerals giving rise to bionanocomposites could result in a synergistic effect concerning both the adsorption and the absorption properties of these systems. For instance, it has been reported that incorporation of chitosan in soils containing sepiolite enhances the flocculation of microalgal cells (*Microcystis aeruginosa*) present in freshwaters [62,217,218]. Sepiolite-cellulose pastes have been used for the salts removal from building stones, facilitating the ions (eg., Na<sup>+</sup>, Mg<sup>2+</sup>) migration, being the treatment of choice for highly porous stones containing large pores when the associated salts are very soluble [219].

The capacity of adsorption of water and water solutions can be extraordinarily elevated leading to “superabsorbency” ability. Synergistic effect in sepiolite and palygorskite nanocomposites containing polyacrylic acid (PAA) and polyacrylamide (PAAm) and their derivatives have been explained by the disaggregation and dispersion at the nanometer scale of those silicates within the polymers [11]. Recently, superabsorbent composites based on sepiolite, CMC, and a copolymer of PAA and PAAm has been applied for the control of the relative humidity in places requiring specific environmental conditions (museums, galleries), thanks to its high moisture absorption capacity and fast response to humidity changes [58].

Biopolymers such as starch, chitosan or guar gum have been associated with PAA and PAAm to develop more ecofriendly and biodegradable superabsorbents. Here again, the incorporation of fibrous clays has proven to enhance both water absorbency and water absorption rate of these systems as reported for guar gum-g-PAA [220] or starch-g-PAAm [55]. Interestingly, guar gum-g-PAA/palygorskite superabsorbents also exhibit good water absorbency after reswelling cycles (Fig. 7), which could be of interest for applications in

agriculture and horticulture [220]. Alternatively, modification of the clay may also change water absorption properties as reported for chitosan-g-PAA/sepiolite composites [221]. Acid treatment of sepiolite provokes lixiviation of  $Mg^{2+}$  ions from the silicate structure and further treatment with metal salts, for instance  $AlCl_3$ , results in modified clays in which the presence of multivalent ions can act as crosslinker of the polymeric network enhancing absorbency and improving swelling rate and water-holding capacity. The incorporation of biopolymers can be also of interest for developing other functional materials for uses in environmental remediation. This is the case of CMC-g-PAA/palygorskite bionanocomposites in which the presence of negatively charged sites in the modified-cellulose chain allows their application in the removal of heavy metal ions like Pb(II) [57]. In a similar way, the presence of protonated amino groups in chitosan-g-PAA/palygorskite bionanocomposites confers to these superabsorbents materials the possibility of being applied in removal of cationic pollutants in water as for instance Cu(II) [222], Hg(II) [223], or cationic dyes, such as methylene blue [224]. In this last case, it is worthy to mention the high desorption of the dye at pH 2.0 allowing the regeneration and reuse of those clay composites making them of potential interest for dye removal. Similarly, chitosan-g-PAA/palygorskite bionanocomposites prepared via *in situ* copolymerization in aqueous solution showed high efficacy for removing  $NH_4^+$  ions in synthetic wastewater [225]. These are promising results considering the ever-increasing global environmental problem in view of ammonium nitrogen coming from disposal of nutrients (N and P) of water plants or indirectly from agriculture and leaching from sludge deposited in landfill and field. Alternative applications are searched in other strategies, as it is the case of alginate-g-poly(acrylic acid-co-acrylamide)/humic acid systems [226]. In this case, a mixture of palygorskite with urea and  $KH_2PO_4$  is combined with alginate and further coated with alginate-g-poly(acrylic acid-co-acrylamide)/humic acid superabsorbent polymer giving rise

to multielement compound fertilizer granules. These materials are partially degradable showing slow release properties that can be applied in agriculture contributing to alleviate environmental pollution.

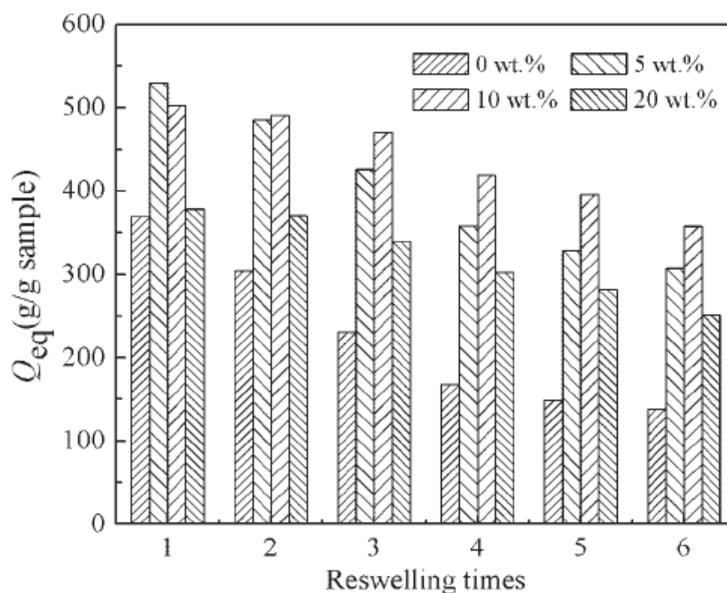


Fig. 7. Evolution of water absorbency properties in guar gum/palygorskite bionanocomposites at different clay loading as a function of reswelling times. (Reproduced with permission from [220]. Copyright 2008, Wiley)

Chitosan can be easily assembled to sepiolite and palygorskite from diluted acid solutions due to hydrogen bonding interactions with silanol groups on the surface of the silicate and also electrostatic interactions as the polymer presents amino protonated sites that can compensate the silicate charge [59]. As the ion-exchange capacity of sepiolite is much lower than that of smectites (approx. 15 mEq/100) these bionanocomposites present free protonated amino groups. Thus, chitosan-sepiolite, as well as chitosan/palygorskite bionanocomposites, have been used as component in electrochemical devices [59] or associated with negatively charged species including dyes for photoprotection of microbial

biocontrol agents [63], respectively. Interestingly, Zou and co-workers [227] observed that the adsorption of Fe(III) and Cr(III) ions on the bionanocomposites is significantly higher than on chitosan or palygorskite alone. Adsorption isotherms fit to a Freundlich model, indicative of multilayer adsorption, which could account for the observed synergistic effect. Other interesting applications in environmental remediation include the use of chitosan-palygorskite materials in the uptake of U(VI) from aqueous solutions [228]. The calculated thermodynamic parameters pointed out that this is a feasible, spontaneous, and endothermic process. In contrast, adsorption of U(VI) by composites prepared by assembly of alginate to a mixture of sepiolite, CaSO<sub>4</sub> and calcined diatomite earth (Kieselguhr) is a spontaneous but endothermic process favored at high temperatures [229]. However, the kinetic of the adsorption process is rapid and the composite shows a high capacity for uranium retention, which could be relevant in view of usage as alternative to other more expensive sorbents commonly employed. Green macro marine algae *Ulva sp.* has been associated with sepiolite to be used as adsorbent of U(VI) [230]. This study does not analyze the nature of the *Ulva sp.*-sepiolite interaction but interestingly experimental evidences that treatment with HNO<sub>3</sub> allows an almost complete uranium recovery. As novel alternatives in the use of clay-based biohybrids for environmental applications, Hrenovic and co-workers [231] reported the stabilization of phosphate-accumulating bacteria on sepiolite. *Acinetobacter junii* is immobilized on the surface of sepiolite fibers by extracellular substances (Fig. 8) and remains metabolically active, being able to remove phosphate from synthetic wastewater. In order to enhance biocompatibility, sepiolite can be modified with polysaccharides (e.g., chitosan) [232] and processed as foams, allowing the colonization of algal and cyanobacterial cells that are able to capture and transform CO<sub>2</sub> into O<sub>2</sub>. Also of special relevance is the possibility to use supported lipid membranes in which organophilic environments are present and may show

changes in surface hydrophilicity of the hybrid material, which can be used as sequestration agent for biological species. In this way, sepiolite-PC biohybrids have been tested in the removal of mycotoxins, such as aflatoxin B1 (AfB1) [72]. AfB1 is adsorbed following a Langmuir-type isotherm reaching adsorptions of about 1440 mg/100 g, therewith demonstrating a superior mycotoxin sequestration capacity than commercial alkylammonium sepiolites and being at the same time less harmful to the environment.

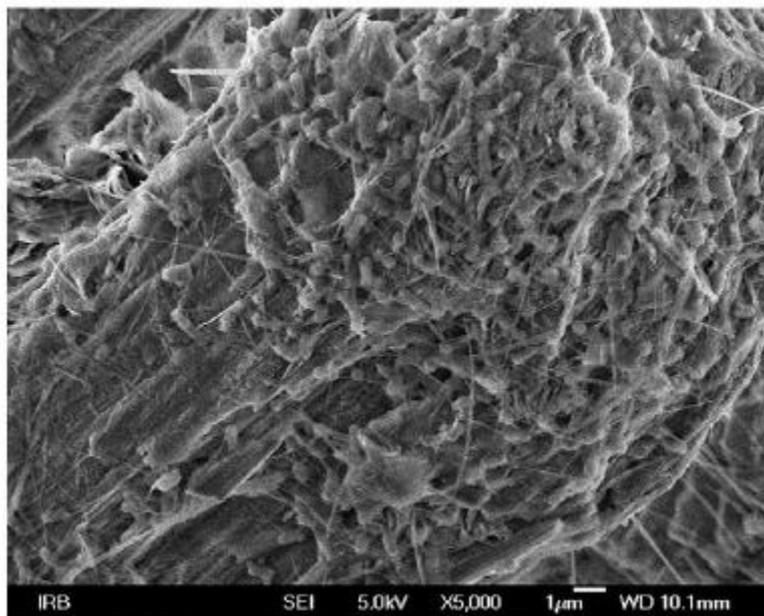


Fig. 8. SEM image of *Acinetobacter junii*, immobilized on sepiolite (Reprinted with permission from [231]. Copyright 2010 Elsevier)

## 6. Bionanocomposites as source of supported carbonaceous materials

Clay minerals can act as templates for the production of carbonaceous materials according to the pioneering works by Kyotani and co-workers [233-235]. Different types of clay minerals, i.e. from layered silicates of the kaolinite and smectite type to fibrous

sepiolite, have been used as porous substrates to produce nanostructured carbon-silicate materials using a large number of molecular and polymeric organic precursors [236]. Although the carbon-clay materials may have interest *per se*, most of the carbon-clay precursors are used as source of nanostructured carbons that show electrical conductivity, being useful for diverse applications as electrode materials such as secondary battery electrodes and supercapacitors [237].

Only few reports on the use of the carbon-clay intermediates based on sepiolite without removal of the silicate substrate have been reported, though the presence of the clay substrate may provide of additional advantageous features [238-240]. The possibility to prepare supported graphene-like materials from sucrose and other natural resources showing interesting characteristics, such as simultaneous conducting behavior together with chemical reactivity and textural features provided by the silicate framework, open the way for diverse high-performance applications [241]. Bionanocomposites based on sepiolite have been recently reported as precursors in the generation of supported graphene materials following the so-called “sweet” way and “jelly” way, respectively (Fig. 9). The first one uses sepiolite-caramel bionanocomposites prepared by thermal treatment of sucrose and sepiolite mixtures, which are transformed at ca. 800 °C in absence of oxygen into graphene-clay materials showing electrical conductivity at room temperature in the  $10^{-2}$  to  $1 \text{ S cm}^{-1}$  range [242] that can be used as electrodes in rechargeable Li- batteries [240]. Interestingly, new chemical functions can be introduced in these graphene-clay materials by grafting of diverse organoalkoxysilanes [239] or by assembly to biopolymers provided of ionizable sites (e.g. alginate) [242], giving rise to modified electrodes for electrochemical ion-recognition.

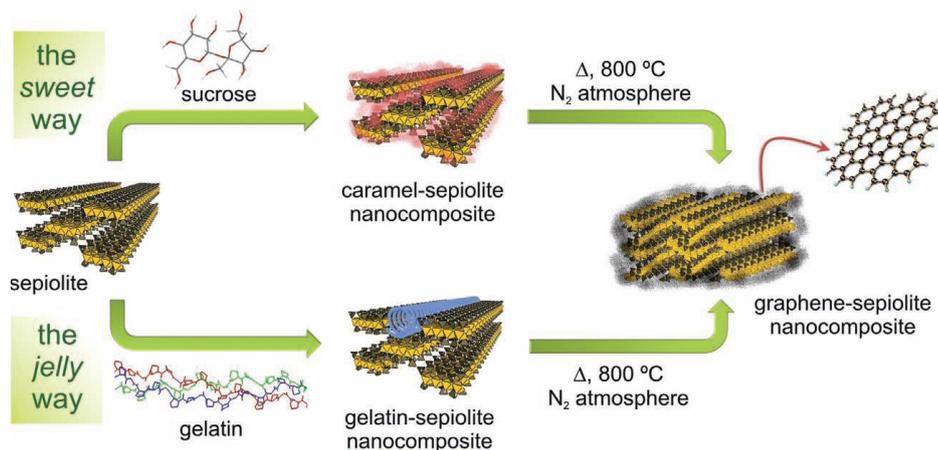


Fig. 9. The sweet way and the jelly way, two synthetic pathways for the preparation of graphene supported on fibrous silicates. (Reproduced with permission from [241]. Copyright 2011, Wiley-VCH Verlag GmbH & Co. KGaA).

The deconvoluted C1s peak in the XPS spectrum of thermally treated caramel-clay samples shows that the main contribution corresponds to C=C bonds, which are present as an intense, sharp, and very narrow component of graphene-like material. Raman spectrum confirms the formation of an extended conjugated system through the G-band at  $1595\text{ cm}^{-1}$  ( $\text{sp}^2$  carbon) and the D-band at  $1360\text{ cm}^{-1}$  ( $\text{sp}^3$ -hybridized carbon). Using gelatin as starting precursor following the “jelly way”, results in supported N-doped graphene materials as it derived from a nitrogen containing biopolymer, which could be useful to improve the electrical conductivity of the resulting materials. In this case, the Raman and the XPS spectra show similar features than graphenes produced from sucrose. In addition, these supported graphenes can be easily conformed as foam materials by freeze drying technique exhibiting good electrical conductivity and macroporosity useful for diverse advanced applications [243].

Comparable results have been obtained using palygorskite instead of sepiolite in the formation of graphenes from caramel-silicate precursors [244]. So, the procedure above discussed represents a cheap and eco-friendly process based on fibrous clays

bionanocomposites that opens the way to regular production of supported graphene-like materials for diverse applications.

## **7. Bionanocomposites as components of sensor devices and bioreactors**

The use of fibrous clays in the development of electrochemical sensors, and enzymatic reactors has been reported since several decades ago. In earlier works, pristine fibrous clays were incorporated as active component of carbon paste electrodes for direct determination of drugs in biological samples, profiting from their sorption properties [245]. In a similar way, fibrous clay-based bionanocomposites provided with suitable functionality may also become the active part of electrochemical sensing devices. This was the case of chitosan-sepiolite bionanocomposites that showed a variable anion-exchange capacity depending on the amount of the positively charged chitosan chains assembled to the clay fibers, which were incorporated in carbon paste electrodes [59]. The resulting sensors were tested for determination of ions in aqueous solution by direct potentiometry, and alternatively this technique could be used for a quick evaluation of the ion-exchange ability of the tested bionanocomposites materials.

As detailed in Section 6, caramel-sepiolite bionanocomposites can be used as intermediate compounds for the development of carbon-clay materials after thermal treatment. The resulting materials can be easily functionalized by grafting of organoalkoxysilanes bearing the desired groups, through covalent linkage with the silanol groups present at the external surface of the sepiolite [239,240]. The great advantage of the thus derived carbon-silicate materials is their multifunctionality, since they offer simultaneously electronic conductivity and ion-exchange ability, being able

to play the roles of sensing phase and electronic collector of the chemically modified electrode at the same time.

In view to develop electrochemical biosensors, the fibrous clay palygorskite has been used as biocompatible matrix for immobilization of proteins and enzymes [246,247] or even bacteria [248]. In these examples, the bio-doped palygorskite membranes were casted on the electrode surface profiting from the film-forming ability of the clay. The clay fibers can provide the biological elements with a protective support, since the confinement within the inorganic matrix may help to prevent the irreversible deformation of the protein structure. As a consequence, the preservation of the biological activity results beneficial to enlarge the stability of the system, allowing the development of biosensors and bioreactors that show a long-term performance. In the case of bioreactor applications, the association with the clay fibers also contributes to make easier the recovery of the biocatalysts for reusing them in consecutive cycles. However, some enzymes can undergo a loss of activity due to strong interaction with the silicate fibers, which can produce the blockage of the amino acids involved in catalysis or the disruption of the three-dimensional structure of the protein. To overcome this drawback, a new approach by Wicklein et al. [73] has proposed the previous modification of sepiolite with phospholipids, forming a biomimetic bilayer for the immobilization of the cytoplasmic enzyme urease (URE). The resulting biohybrid was successfully evaluated as the sensing part of a biosensor for urea determination (Fig. 10), which was operative over several months. This high stability was attributed to the presence of the lipid bilayer covering the clay surface, which avoided the denaturation of urease in contrast to that observed in pristine sepiolite.

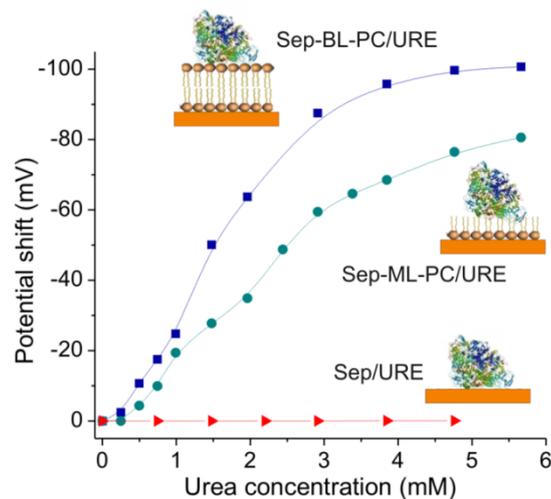


Fig. 10. Biosensors based on the immobilization of urease on pristine sepiolite (Sep/URE) and on phosphatidylcholine-modified sepiolite, with the phospholipids in monolayer (Sep-ML-PC/URE) or in bilayer (Sep-BL-PC/URE) configuration. (Adapted from reference [73]).

Similar problems of bioactivity reduction due to the strong adsorption of the enzymes to the clay fibers can be found in bioreactor applications, where fibrous clays are also used as support of enzymes or even bacteria due to their biocompatibility and high specific surface area [231,249]. This is the case of a pig pancreatic lipase associated to sepiolite whose efficiency after immobilization is reduced to about 42% of the value determined for the free enzyme thus, diminishing the effectiveness of this bioreactor for biofuel production [249]. As already mentioned, the modification of the fibers surface with organic molecules or polymers may help to overcome this problem, preserving the activity of the immobilized enzymes. Thus, the biomimetic lipid bilayer assembled to fibrous clays may be advantageous for bioreactors development, similarly to that previously described for the immobilization of urease on sepiolite [73]. The supported phosphatidylcholine bilayer and monolayer membranes offered a compatible environment for the immobilization of the membrane-bound enzyme cholesterol

oxidase (COx), yielding highly stable, functional biohybrid materials. These COx-biocatalysts were successfully tested in a stirred batch reactor setup, showing preservation of the enzymatic activity under process conditions and throughout recovery and recycling [73]. Previous modification of the sepiolite fibers with ferrofluids introduced magnetic properties in this type of bio-hybrids that made the recovery of the biocatalysts easier [250]. In this case, the presence of a layer of oleic acid coming from the ferrofluid was used to create a hybrid bilayer by association of PC molecules. The mixed oleic acid/PC clay-supported membranes were proven to be also suitable for accommodation of COx, preserving its enzymatic activity and ensuring the recyclability and reusability of the prepared biocatalysts, which can be simply recovered after each cycle with the help of a magnet.

The successful application of biopolymer-clay nanocomposites based on other types of clays in the development of enzymatic sensors or reactors [251,252] support the potential use of fibrous clays-based bionanocomposites for analogous purposes. Their biocompatibility and suitability as immobilization matrix for other biological entities has been proved by association of different types of microorganisms. Some recent examples are the xanthan-sepiolite material used as virus carrier in vaccine formulations [64] mentioned in Section 4.3., or the use of a chitosan-sepiolite foam as support of living microalgal cells of *Chlorella vulgaris* and *Anabaena sp.*, which showed enhanced viability and were able to proliferate and colonize the macroporous support [232]. Similarly, chitosan-palygorskite bionanocomposites modified with photoprotective anionic dyes served as support of fungal spores used as microbial biocontrol agent, increasing their stability against solar UV radiation [63]. However, to the best of our knowledge, the use of biopolymer-clay nanocomposites based on fibrous clays as supports for enzyme immobilization has not yet been evaluated. This opens new

expectative within this field of research, with possibilities to develop sensor devices and bioreactors with enhanced sensitivity and long term-stability.

## **8. Concluding remarks**

Biohybrid nanostructured materials constitute a research field of growing interest due to the large field of potential applications dealing with topics from biomedicine to environmental protection. In the past, most part of nano-hybrids were produced by the combination at the nanometric scale of natural polymers and layered inorganic solids, but recently bionanocomposites based on sepiolite and palygorskite fibrous clays become a highly attractive field in view of new applications. The particular physical and chemical properties of this special type of clay minerals allow their assembly to biopolymers of different nature from polysaccharides, in which the presence of many OH groups in either uncharged or charged with positively or negatively charged sites favors their linkage, to zwitterionic species, such as proteins or PC, or even negatively charged DNA chains. The conformation of the biohybrids as films, foams or biomimetic interfaces opens way to many new potential applications as so-called advanced materials with either functional or structural properties. Examples of novel applications of these bionanocomposites are their use as green plastics, active phases of ion-sensors and biosensors, among other uses and perspectives.

The use of sepiolite and palygorskite as nanofiller in the preparation of green nanocomposites becomes of growing importance as the presence of OH groups on the fiber surface favors either the direct assembly to many biopolymers or the possibility to introduce appropriate functionalization to make them more compatible with the polymer matrix, which results in a significant improvement of the mechanical properties.

Bionanocomposites also show potential interest in regenerative medicine and as new substrates to immobilize compounds of biological origin and even living entities. For instance, the bioactivity showed by virus-bionanocomposites prepared from sepiolite-polysaccharides allows their application as effective vaccine adjuvants, which can increase the immune response against influenza viruses compared to conventional vaccines based on the virus alone. Also in biomedicine these nanostructured materials are useful as drug delivery systems or as carrier for DNA vectorization. Palygorskite and sepiolite bionanocomposites result also especially attractive for environmental protection in view to reduce hazardous species and, in many cases, profits of a synergistic effect that confers adsorption and absorption properties to these systems. Moreover, as these materials are partially degradable they can be also used as slow release systems in agriculture contributing to alleviate environmental pollution. On the other hand, the possibility to assemble phospholipids to these fibrous clays results in biomimetic supported lipid membranes that can be used as biocompatible matrix for immobilization of enzymes or bacteria without loss of activity. Finally, as a new field of application, sepiolite-based bionanocomposites have been used as green source to prepare supported graphene-like materials simultaneously providing a conducting behavior together with chemical reactivity and textural features, which can be of interest for diverse high-performance applications.

More deep research in this field is necessary to accomplish the promising future expected to bionanocomposites based on fibrous clays. To present, most part of research was devoted to pristine sepiolite and more scarcely to palygorskite, in both cases without chemical modifications of the silicates. Controlled changes of their functionality, together with implication of new biopolymers –or biopolymer blends– as well as new strategies for the preparation and conformation of this type of

bionanocomposites will be of great interest in view to develop novel bionanohybrids with improved characteristics as advanced materials.

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