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Chemical modification of Halloysite nanotubes for controlled loading and release

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Clay minerals have been used for medical purposes from ancient times. Among them, halloysite nanotubes, an aluminosilicate of the kaolin group, is an emerging nanomaterial which possesses peculiar chemical characteristics. By means of suitable modifications, as supramolecular functionalizations or the covalent ones, it is possible to obtained novel nanomaterials with tunable properties for several applications. In this context the covalent grafting of suitable organic moieties on the exernal surface or in the halloysite lumen has been exploited to improve the loading and release of several biological active molecules. The resulting hybrid nanomaterials have been applied as drug carrier and delivery systems, filler for hydrogels, in tissue regeneration and in gene delivery field. Furthermore the loading and release of specific molecules have been also investigated for environmental purposes. This review summarizes the main developments in the halloysite modifications in the last 20 years with a particular attention to the past two years.

1. Introduction

Clay minerals are layer-type aluminosilicates that are formed as products of chemical weathering of other silicate minerals at the earth's surface.[1] These minerals have a platy morphology because of the arrangement of atoms in the structure. The different arrangement of the tetrahedral and octahedral sheets allowed us to classify the clays in three categories: 1:1, 2:1 and 2:1:1 phyllosilicates. Due to the fact that clay minerals are available in large amount at low cost they have been used as raw materials for hundreds of industrial applications such as in engineering and construction applications, environmental remediation, food processing and so on (Figure 1).[2]

Clay minerals have been used in medicine since ancient times. There are traces that minerals have been used for curative applications since prehistory; *homo erectus* used ochres mixed with water and different type of mud in order to cure wounds. The first written reference about the use of ''stones'' as "curative powers", dates from Roman times. Several years later, when *Pharmacopoeia* appeared in Renaissance, clays have been classified among other drugs. The different minerals for medicinal uses and regulations were listed and this was also the first mineralogical classifications. Clays are an important, widely abundant, low-cost and no toxic class of materials with unique properties such as intercalation, swelling, ion-exchange and adsorption properties. Normally in the USA clay minerals are used as excipients in commercialized pharmaceutical products and indicated as "Inactive Ingredient".

Drug molecules are encapsulated in clay minerals to modify the rate, the time and to target the site of drug release. Moreover, this strategy can be useful to protect drugs against aging due to chemical and enzymatic degradation. On this basis, a new concept of "excipient" is generated for clay minerals as they are not inert fillers but, instead, they can have a functionality providing targeting release, prevention or reduction of side effects and increasing the product shelf-life.

Depending on the clay minerals physico-chemical properties it is possible foreseen several formulations which can improve the drug bioavailability and control the efficiency of the dosage form.[3]

For example, Montmorillonite was used to deliver Sildenafil,[4] Aripiprazole,[5] and Piroxicam.[6]

Recently, among the plethora of clay minerals, great attention is paid to use the kaolin minerals as excipient in pharmacological preparations for both topical and oral administration (Figure 2a). In this context, halloysite an aluminosilicate clay of the kaolin groups has been becoming attractive for the scientific community as proved to the more and more increasing number of publications. It is noteworthy how in the last year, the number of the patent about halloysite is greater than that of scientific papers, as consequence of an increasing interest also for industrial purposes (Figure 2b).

In this review, we summarize the development in the halloysite field in the past 20 years as far as is concerning the covalent or supramolecular functionalizations of halloysite

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surfaces and the main applications in the loading and release of molecules.

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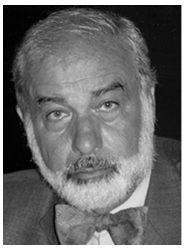
Giuseppe Cavallaro is post doc at the Department of Physics and Chemistry, University of Palermo, Italy. He was Research Associate at Institute of Micromanifacturing, Lousiana Tech Univesity, (USA) and Institut fur Chemie, Technische Universitat Berlin (Germany). His research activities focus on nanoclays and polymer/nanoparticle interactions. He is author of 45 pubblications in peer-reviewed international journals.

Carmelo Giuseppe Colletti obtained is PhD at the University of Palermo in the 2018 under the supervision of Dr. Riela. In 2016 he spent nine months at the Institute for Micromanufacturing, Louisiana Tech University as a research associate under the supervision of Dr. Yuri Lvov. Specialising in supramolecular chemistry, Dr. Colletti focuses on applications in enhancing nanomaterials like Halloysite clay nanotubes. He has published papers in modifying and grafting the external surface of halloysite nanotubes with molecules like curcumin, cyclodextrin and have also designed catalyst systems.

Giuseppe Lazzara received his PhD degree in Chemistry at University of Palermo, Italy, in 2007. He was Postdoc at the Chemistry Department, Lund University (Sweden). Lazzara became associate professor at the Department of Physics and Chemistry, University of Palermo (Italy) in 2015. His research activities focus on smart nanomaterials, nanoparticles and polymer/nanoparticle interactions. He is involved in several projects on halloysite clay nanotubes for drug delivery and conservation of Cultural Heritage. Lazzara has more than 100 publications in peer-reviewed international journals.

Stefana Milioto received the Master Degree in Chemistry at the University of Palermo in 1985. She Defended the PhD thesis in Chemical Sciences in 1989. Then she was Research Scientist (until 1998) and associate professor (until 2001) in Physical Chemistry. At the present she is Full Professor in Physical-Chemistry at the Department of Physics and Chemistry of the University of Palermo. Her scientific interest deals with the physico-chemical studies of eco-compatible nanostructures (self-assembled structures as well as solid nanoparticles) functionalized for application in the field of Cultural Heritage, drug delivery, and so on. She has more than 130 publications in peer-reviewed international journals.



Renato Noto obtained his chemistry degree from the University of Palermo in 1970. He has been Full Professor of Organic Chemistry since 1990. He began his research activity in the field of physical organic chemistry. Then his interest moved to stereocontrolled synthesis of functionalized heterocyclic rings and to studies on inclusion equilibria of aromatic molecules in cyclodextrins. Recently he has addressed his attention to the behavior of organic salts as ionic liquids and/or gelators and to use of functionalized halloysite nanotubes as catalytic system or drug carrier system. He is author of more than 220 publications and five reviews.

Serena Riela since 2002 is an Assistant Professor at the University of Palermo. She received her Ph.D. from the University of Bologna in 2000, under the supervision of Prof. D. Spinelli. From April 2001, she was post-doctoral researcher at the Organic Chemistry Department of the University of Palermo. She spent one year at University of California Los Angeles (UCLA) under the supervision of Prof J. F. Stoddart and eight months at the University of Bordeaux 1 under the supervision of Prof. A. Castellan. Her current interests are focused on chemical modification of halloysite nanotubes and their applications in catalysis, drug delivery, bioremediation and so on. Riela is author of about 70 publications in peer-reviewed international journals.

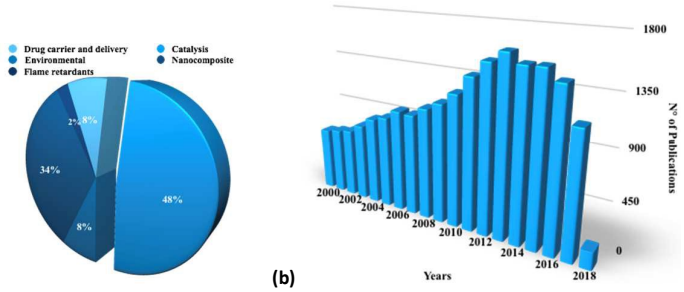


Figure 1. Comparison of (a) application fields of "clay minerals"; (b) the annual number of scientific publications and patents related to the term "clay minerals". The data reflect the past 20 years. (Data analysis of publications was done using the SciFinder Scholar search system with the term "clay minerals", as on January 2018).

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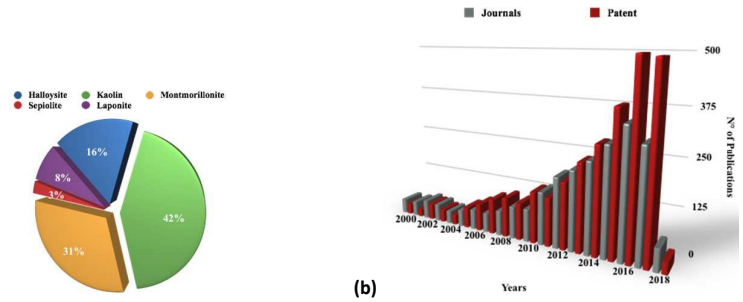

Figure 2. Comparison of the number of scientific publications on the (a) 'halloysite', 'kaolin', 'montmorillonite', 'sepiolite' and 'laponite' terms with the term 'drug'; (b) distribution (%) of scientific publications in 'patent' and 'journal' for halloysite. Data analysis of publications, as on January 2018, was done using the SciFinder Scholar search system using as 'Document type' the 'Journal' and 'Patent', respectively.

2. Halloysite nanotubes

Halloysite nanotubes (HNTs), with chemical formula $Al_2Si_2O_5(OH)_4 \cdot nH_2O$, are novel 1D natural inorganic clay nanotubes belonging to kaolin group. It is a dioctahedral 1:1 clay mineral present in soils. It is formed by weathering of several types of igneous and non-igneous rocks thus it can be found mainly in wet tropical and subtropical regions and weathered rocks. Of course, each deposit is characterized by different purity grade, characteristic sizes and hydration state.

The term 'halloysite' was employed for the first time by M. Berthier in 1826 and derived from Omalius d'Halloy, who found the mineral in Angleur, Liége, Belgium.

Halloysite has mainly a hollow tubular structure in the sub-nanometer range with an aspect ratio of ca. 20; the wall is constituted of 10–15 bilayers, with a spacing of approximately 0.72 nm and has a density of 2.53 g/cm^3. The external surface is composed of siloxane (Si–O–Si) groups, whereas the internal surface consists of a gibbsite-like array of aluminol (Al–OH) groups, and Al–OH and Si–OH groups at the edges of the material. The sequence of the layers gives to the tubes Si–O groups at the outer surface, Al–OH groups at the inner surface (Figure 3). They undergo to ionization in aqueous media in an opposite way generating tube with inner and outer surfaces oppositely charged.[6] This charge separation occurs in water within a wide pH range from 3 to 8.[7] Experimentally, the charge separation is predicted by comparing the negative and positive values for electrical ζ–potential of silica and alumina surfaces in water, respectively.

Generally, halloysite has a length in the range of 0.2–1.5 μm, while the inner and outer diameters of tubes are in the ranges of 10–30 nm and 40–70 nm, respectively[8-10] depending on the extraction site and purification processes. In some deposits, indeed, there were found halloysite tubes with length up to 3–5 μm but in the size distribution curve they have a minor fraction. However, these shorter tubes are the most attractive from a biological point of view since they are more suitable for composites with sustained delivery of chemicals and drug formulations with regards to the longer ones. Besides, clay tubes 1 μm in length may have an additional advantage

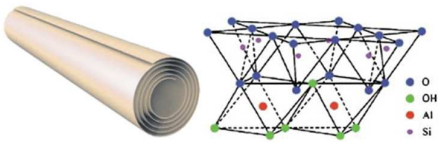

Figure 3. Schematic representation of the rolled structure of halloysite (left) and schematic illustration of the crystalline structure of halloysite (right).

because it is a safe dimension for macrophage removal of the nanoparticles from living organisms.[10]

Unfortunately, in the human body are not present the biological mechanisms for the aluminosilicate degradation and therefore the halloysite nanotubes cannot be used for injectable drug delivery since it might cause thrombosis. For this reason, currently, the use of halloysite nanomaterials is limited for the development of creams, implants which can afford a slow release of the loaded drugs for external medical treatment.

In this context there are several studies which deal the biocompatibility of halloysite. It has been shown that the nanoclay is non-toxic to cells and,[23,24] more in particular, in the case of mammalian cells has been found that this nanoclay is less harmful than the common sodium chloride.[25] Besides, halloysite tubes may have an appropriate dimension for macrophage removal from living organisms.[22,26] Moreover, the interaction of halloysite nanotubes with microscopic algae *Chlorella pyrenoidosa* was also investigated. Lvov *et al.* demonstrated that there was no penetration of the nanomaterials into cell interior due to electrostatic interactions between the cell wall surface and halloysite.[11] It was also reported that halloysite nanotubes were safe for fresh water ciliate protist *Paramecium caudatum*[12] and it exhibits no toxicity towards *Escherichia coli* bacteria [13] as well as in yeast cells.[8]

A recent *in vivo* study[14] has shown that by feeding the free living nematodes (worms) *Caenorhabditis elegance* with halloysite, the nanotubes are only localized in the intestines of the worms and none of them were detected outside the intestines of the nematodes.

Recently, the halloysite research has been focused to perform studies on the cytotoxicity of halloysite for its oral use.[15] In particular, studies performed on the hepatocarcinoma cells HepG2 and the colorectal carcinoma cells HCT116,

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representing respectively the cells of the first major organ where the nanomaterials are generally localized and accumulated after absorption and the epithelial lining of the major absorptive site for drugs administered via oral route showed that the nanotubes are safe materials.

Therefore, it is possible to foresee application of halloysite as filler for oral formulation composites since its systemic absorption is prevented.

In vivo tests conducted on chickens and piglets have shown that by fed the animals with halloysite it is also possible to treat them by removing mycotoxins such as zearalenone and deoxyvalenol sometimes occurring in grain feed.[16]

The oral toxicity of halloysite was also investigated by Hu *et al.*,[17] who studied the hepatic toxicity of purified halloysite in mice via oral route. It was found that the oral administration of halloysite, stimulated the growth of the mice at low dose (5 mg kg^{-1} per body weight (BW)), but inhibited their growth at middle or high dose in a dose-dependent manner (from 50 to 300 mg kg^{-1} BW). The observed toxicity was probably due to an accumulation of Al or Si in the liver. To assess this, the Al and Si contents in hepatic tissues were studied by ICP-AES after 30 days repeated administration of halloysite. The authors find that the Si content remains almost unchanged, whereas when halloysite is administered in high dose, the Al content is significant increased inducing oxidative stress as a consequence of hepatic dysfunction and histopathological abnormalities. Thus, this study highlights that the oral administration of halloysite should be controlled ensuring the oral administration limit at ca. 20 mg kg^{-1} BW.

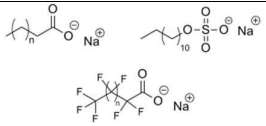
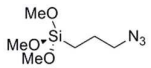
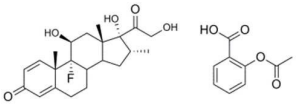
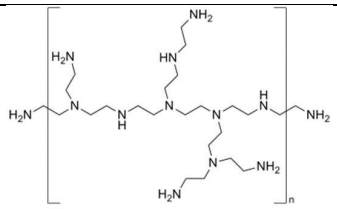
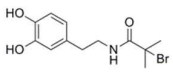
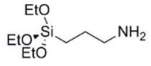
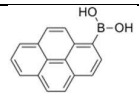
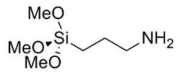
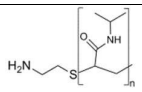
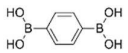
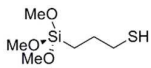
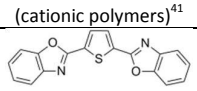
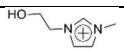
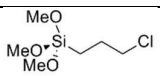
In addition, a study on *Raphanus Sativus* L has demonstrated that halloysite possesses positive effect on growth of seeds, showing no phytotoxic effects.[18]

3. Modification of halloysite surfaces

Among the different phyllosilicate nanomaterials (silicate based layer structure) such as kaolin and montmorillonite, halloysite has distinct advantages. Indeed halloysite possesses an empty lumen which can be loaded with suitable molecules or alternatively, the molecules can be absorbed onto the external halloysite surface.

Furthermore, the possibility to both load, for example, drug into the inner lumen and graft it onto the outer surface offers distinctive additional advantages, i.e. synergism between drugs with different biological properties. Therefore, in the remaining sections we focused on the introduction of chemical modifications of halloysite surfaces both *via* supramolecular interactions and *via* covalent grafting of different kind of molecules. Table 1 summarizes the different methods adopted for the halloysite surfaces functionalization.

Table 1. Overview of the different halloysite functionalization methods.

Supramolecular Functionalization		**Covalent Modification**	
Inner surface	***Outer surface***	***Inner surface***	***Outer surface***
 Anionic surfactants[19-21]	 Alkyltrimethyl ammonium bromide (Cationic surfactants)[22]	 Octadecyl posphonic acid[23]	 3-azidopropyltrimethoxysilane[24]
 Dexamethasone,[25] Aspirin[26] (Drugs)[27, 28]	 Poly(ethyleneimine)[29, 30]	 2-bromo-N-[2-(3,4-dihydroxyphenyl)ethyl]-isobutyryl amide[31]	 3-aminopropyltriethoxysilane[26, 32, 33]
Lipase, Glucose oxidase, Albumin Laccase and Pepsin (pH < pI) Binase Co-enzymes or enzymes[34-36]	Lipase, Glucose oxidase, Albumin Laccase and Pepsin (pH > pI) Enzymes[36]	 1-pyrenyl boronic acid[37]	 3-aminomethyltrimethoxysilane[38-40]
Pectin Alginate Anionic polymers[41]	 Chitosan Amine terminated PNIPAAM[42] (cationic polymers)[41]	 1,4-phenylenebisdiboronic acid[43]	 3-mercaptopropyltrimethoxysilane[44, 45]
			

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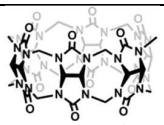
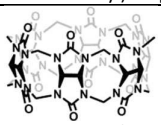
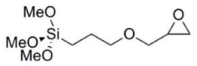
Carbon dots[46]	2,5-bis(2-benzoxazolyl)thiophene[47]	3-(2-hydroxyethyl)-1-methyl-imidazolium[48]	3-chloropropyl-trimethoxysilane[49]
 Cucurbituril[50, 51]	 Cucurbituril[50, 51]		 γ–glicidoxypropyltrimethoxysilane[52, 53]

Supramolecular

The selective modification of halloysite can be easily achieved by using ionic molecules, which selectively interact with the halloysite surfaces though electrostatic interactions.[19, 54] Due to their different chemical composition, the halloysite inner and external surfaces are positively and negatively charged, respectively, in a wide pH interval (from 3 to 8).[7] Accordingly, molecules with a positive charge are adsorbed into the cavity, while the negatively charged compounds interact with the halloysite surfaces. The selective interactions between ionic molecules and halloysite interfaces affect the halloysite dynamic behavior as well the surface charge. Literature[21, 55] reports that the adsorption of anionic surfactants onto the inner surface induces an increase of the negative ζ–potential of halloysite as a consequence of the neutralization of the positive charges of the halloysite lumen. The confinement of the anionic surfactants did not influence the aqueous diffusion coefficient of halloysite indicating that the modified nanotubes exhibited a single diffusive mobility. Similar observations were detected for hybrid materials based on halloysite and anionic polymers, such as pectin[54] and polystyrene sulfonate.[56] Stable Pickering emulsions were obtained by modification of halloysite with anionic sodium dioctyl sulfosuccinate.[57] Contrary to the anionic surfactant/halloysite hybrids, the functionalization of the outer surface with cationic surfactants determined the neutralization of the negative charges of halloysite.[22] As a consequence, an inversion of the halloysite ζ–potential was observed in composite materials formed by halloysite and the cationic hexadecyltrimethylammonium bromide.[22] In addition, the adsorption of cationic surfactants onto the external surface reduced the mobility of the nanotubes in water because of the hydrophobic interactions between the alkyl chains that favored the formation of aggregates.[22] The functionalization degree of the halloysite surfaces was significantly influenced by the head group of the adsorbed molecules. Within the anionic surfactant/halloysite hybrid, sodium dodecanote evidenced a much larger loading with respect to that of sodium dodecylsulphate.[58] Small angle neutron scattering (SANS) curves (Figure 4a) evidenced that the sodium dodecanoate/halloysite hybrid presents a peak at q (magnitude of the scattering vector) = 1.79 nm^{-1}. According to the Bragg law, this signal corresponds to a characteristic length of 3.5 nm that might reflect the formation of multilayers structures or

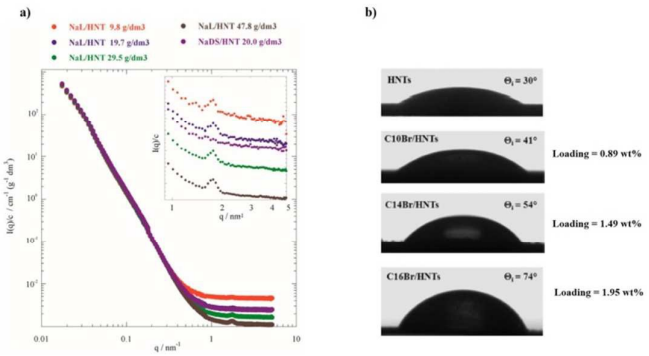


Figure 4. a) SANS curves for dispersions of halloysite modified with sodium dodecanoate (NaL) and sodium dodecylsulphate (NaDS). Inset shows the details of the q range between 0.9 and 5 nm^{-1} in linear intensity scale. Adapted from ref. [58] with permission from American Chemical Society. b) Initial water contact angle for pure halloysite and halloysite modified with alkyltrimethylammonium bromides (C10Br, C14Br and C16Br are decyltrimethylammonium bromide, tetradecyltrimethylammonium bromide and hexadecyltrimethylammonium bromide, respectively). The corresponding surfactant loadings are reported. Adapted from ref. [22] with permission from American Chemical Society.

cylindrical packing of surfactant within the halloysite lumen. This hypothesis is consistent with the surfactant loading (4.5 wt%) and the length of the hydrocarbon chain (1.7 nm).[58] On the other hand, any peaks were observed in the SANS curve of sodium dodecylsulphate/halloysite composite in agreement with the monolayer adsorption of the surfactant onto the halloysite inner surface.[58]

Furthermore, the length of the surfactant alkyl chain influences the functionalization degree as observed for sodium alkanoates/halloysite hybrids.[22] Sodium alkanoates with a longer hydrocarbon chain (sodium dodecanoate and sodium tetradecanoate) are organized in complex structures inside the halloysite lumen, while shorter sodium alkanoates presents a monolayer adsorption that can be attribute to the reduced hydrophobic interactions between the alkyl chains of the adsorbed.[21] Anionic fluorocarbons surfactants (perfluoroalkanoates) were selectively adsorbed onto the halloysite internal surface with the aim to generate non-foaming oxygen nanocontainers in aqueous.[20] The surfactant loading values are consistent with a monolayer adsorption for halloysite modified with sodium perfluoroalkanoates. As observed for sodium alkanoates/halloysite hybrids, the increase of the surfactant tail improves the functionalization efficiency of halloysite.[20] Similarly, the modification of the halloysite outer surface with alkyltrimethylammonium bromides was enhanced by increasing the length of the

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surfactant alkylchain. Interestingly, the hydrophilic/hydrophobic nature of the halloysite interfaces can be tuned by the targeted adsorption of ionic molecules onto the halloysite surfaces allowing to control the halloysite colloidal stability in solvents with variable polarity.[22] The adsorption of anionic molecules (polymers, surfactants, proteins) into the halloysite cavity increased the halloysite colloidal stability in water due to the stronger repulsive interactions between the nanotubes, which possess a larger negative surface charge with respect to the unmodified halloysite.[21, 54] In contrast, the aqueous colloidal stability of halloysite functionalized with alkyltrimethylammonium bromides is lower than that of pure halloysite because of the clustering effect that enhances the sedimentation kinetics.[22] The presence of alkyltrimethylammonium bromides onto the halloysite external surface increased the affinity of the nanotubes towards apolar solvents, such as chloroform and 1-octanol,[22] driving to obtain reverse micelles for water-in-oil emulsions. Within this, hexadecyltrimethylammonium bromide/halloysite reveled efficient in the encapsulation of hydrophilic compounds (such as copper sulphate) within its hydrophilic cavity. It should be noted that the enhancement of the affinity towards apolar solvents is due to the hydrophobization of the halloysite external surface, which was demonstrated by the increase of the initial water contact angle (Figure 4b). As shown in Figure 4b, alkyltrimethylammonium bromides with a longer hydrocarbon chain generate a stronger hydrophobic character of the modified nanotubes because of the larger surfactant coverage. The cationic poly(ethyleneimine) (PEI) was successfully adsorbed onto the halloysite outer surface determining the formation of a polymer monolayer with a thickness of 54 nm.[29] Based on the polymer/halloysite electrostatic interactions, Lvov *et al.*[29] deposited a multilayer structure of PEI onto the halloysite outer surface by using a layer by layer (LBL) technique. Due to the loosely packed structure, the obtained composite could be used to load and, subsequently, release guest molecules.[29] A similar LBL method was used by Han *et al.*[30] for the coating of the halloysite external surface with PEI. The coated nanotubes were investigated as carrier for loading and controlled release of clove bud oil, which is a natural insect-repellant for food packaging applications. The halloysite modification with PEI was explored to obtain emerging nanomaterials for CO_2 capture.[59] It was observed that halloysite loaded with 50 wt% of PEI polymer exhibits a CO_2 adsorption capacity of 2.75 mmol g^{-1}.[59] The adsorption of positively charged poly-(N-isopropylacrylamide)-amine terminated (PNIPAM-NH_2) allowed to fabricate thermosensitive nanocarriers.[42] The presence of the polymer induced a strong enhancement of the halloysite colloidal stability in water on dependence of the temperature as evidenced by the sedimentation kinetics (Figure 5a). Accordingly, the hydrodynamic diameter of the PNIPAM-NH_2/halloysite hybrid showed an increase for temperature > ca. 40 °C, which is the lower critical solution temperature (LCST) of the adsorbed polymer (Figure 5b).

Literature reports that halloysite can adsorb organic molecules *via* electron transferring interaction as observed in the functionalization of the halloysite outer surface with 2,5-bis(2-benzoxazolyl) thiophene (BBT), which is an electrons donor molecule.[47] The nanotubes modified with BBT were filled into polypropylene matrix driving to obtain nanocomposites with enhanced tensile and flexural properties.[47] LBL deposition of polyelectrolytes with variable molecular weight onto the halloysite external surface was employed to prepare modified nanotubes with loading ability towards dexamethasone.[25] The reduction of the porosity in the nanotubes loaded with dexamethasone proved the successful loading, which was equal to ca. 7 vol.% in agreement with the encapsulation of the drug within the halloysite lumen.[25]

It was demonstrated that the positive halloysite cavity is suitable to encapsulate chemically and biologically active molecules with a negative electron density including isoniazid,[60] vancomycin,[61] norfloxacin,[62] salycilic acid,[63, 64] bovine serum albumin, α lactalbumin and β–lactoglobulin.[65] On this basis, the halloysite lumen can act as a nanocontainer for chemical agents, which should be protected by external stimuli, such as photo- or enzymatic degradation processes. Price *et al.*[34] proved that halloysite cavity is efficient in the entrapment and controlled release of tetracycline HCl (a water soluble antibiotic), khellin (a lipophilic vasodilator) and nicotinamide adenine dinucleotide (a co-enzyme). Based on the halloysite geometrical characteristics, the maximum loading of molecules into lumen is ca. 10 vol%. Interestingly, the halloysite inner diameter can be increased by treatment with H_2SO_4 allowing to enhance the potential loading up to ca. 30-40 vol%.[66]

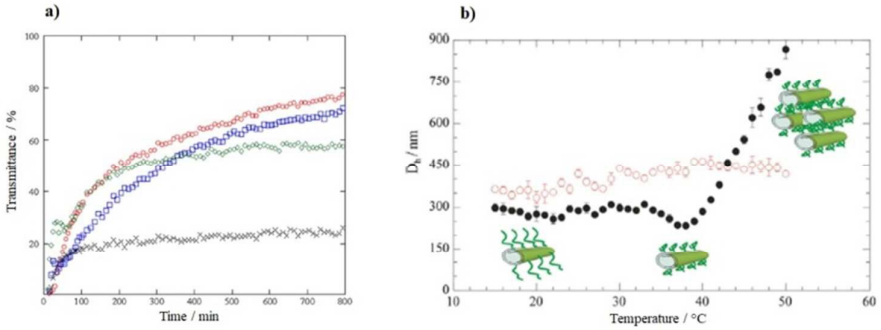

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Figure 5. a) Transmittance at 600 nm as a function of time of halloysite at 15 °C (o) and 45 °C (□) and PNIPAM-NH_2/halloysite (0.5 wt%) at 15 °C (x) and 45 °C (◊) in water above and below the LCST. b) Hydrodynamic diameter for halloysite (empty circles) and halloysite/PNIPAM-NH_2 (filled circles) as functions of temperature. Adapted from ref. [42] with permission from Elsevier.

The incorporation of lipase into the halloysite lumen allowed to fabricate chitosan based membranes with enzymatic activity for lipid decomposition.[35] The coating of the outer surface of the halloysite/lipase composite with cationic lysozyme induced the formation of nanotubes with dual enzyme actions.[35] The effect of pH on the immobilization of several enzymes (laccase, glucose oxidase, lipase, and pepsin) into the halloysite cavity was investigated.[36] It was observed that negatively charged proteins present loading values of ca. 5–7 wt% and their release from the halloysite lumen is extended over time. Glucose oxidase entrapped into the halloysite lumen showed an improved thermal stability as well as an extension of the storage time. All adsorbed enzymes exhibited improved biocatalytic abilities on dependence of the pH conditions. Halloysite cavity was used as container for binase, which is the RNase from *Bacillus pumilus*.[67] Compared to the pure enzyme, the halloysite/binase complex possesses a much stronger anticancer efficiency towards human cancer colon cells because of its ability to uptake on the cells surface as highlighted by fluorescence microscopy (Figure 6). Fluorescent carbon nanodots with antioxidant properties, obtained by the microwave mediated pyrolysis of citric acid in the presence of 1,2-ethylenediamine, were successful loaded into halloysite lumen (Figure 7).[46] The characterizations of the hybrid material confirm the loading of the CDs and the kinetic release test highlighted that by covalent functionalization of the halloysite tubes with silanes, it is possible to slow down the release and therefore the hybrids can be applied in that processes which benefit from a slow release.

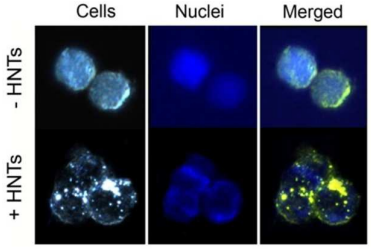

Figure 6. Microscopic visualization of Colo 320 cells after 24 h of incubation with 100 µg/ml of halloysite. Dark-field image of cells (left), fluorescent image (middle), merge image (right). Cells nuclei are stained with DAPI. Adapted from ref. [67] with permission from Frontiers.

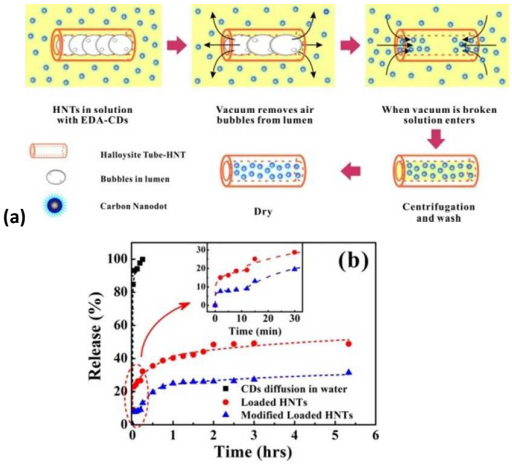

Figure 7. a) Loading Procedure of Halloysite Nanotubes with Carbon Nanodots; b) Cumulative CD release profiles from its solid powder, pristine halloysite, and halloysite encapsulated with silane in water. Reproduced from ref. [46] with permission from American Chemical Society.

Recently, Lvov *et al.*[28] investigated the potential application of halloysite as oral drug delivery system by preparing targeted tablets containing a pharmaceutical excipient with excellent compression properties (Figure 8). In particular, the loading of nifedipine within the halloysite lumen generated suitable nanofillers for the formulation of tablets, which were prepared by blending with microcrystalline cellulose, colloidal silicon dioxide, magnesium stearate and croscarmellose sodium. Remarkably, the confinement of nifedipine inside the lumen prevents its photo degradation. Release experiments showed that the drug can be retained in the tablet formulation for a long time (up to 20 hours).

Similarly, the encapsulation of paclitaxel (an antitumoral drug) inside the halloysite lumen was conducted to slow down its release and, consequently, to increase its therapeutic efficacy.[27] To this purpose, the nanotubes were firstly coated with dextrin[68] or polymeric materials providing a stimuli-responsive coating with tube-end clogging. The obtained complex was used as excipient in tablet formulation that ensures a drug release within 250 h (Figure 9a). Coating of halloysite with poly(methyl methacrylate-co-methacrylic acid (a pH-sensitive polymer) hindered drug release in acidic conditions, while the opposite effect was detected at basic pH conditions (Figure 9b). Coating of halloysite with dextrin allowed for enhancing paclitaxel release in the cancer cells when dextrin was enzymatically decomposed. Both these additional polymeric coatings provided triggered drug release for its higher efficiency. *In vitro* assay to evaluate the cytotoxicity of the halloysite systems towards human lung and liver cancer cells showed a significant antitumoral effect of the halloysite/paclitaxel hybrid.

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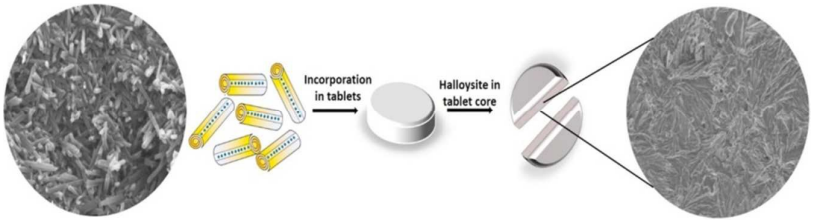

Figure 8. SEM images showing uncompressed drug loaded nanotubes (left) and bound nanotubes (right) compressed in the tablet core. Adapted from ref. [28] with permission from Elsevier.

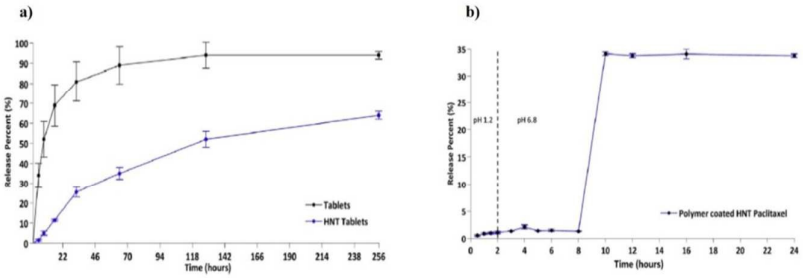

Figure 9. a) Paclitaxel release from tablets containing drug admixed with halloysite as compression excipients and formulation with the drug encapsulated into halloysite. b) pH triggered release of paclitaxel from halloysite coated with poly(methyl methacrylate-co-methacrylic acid at different pH. Adapted from ref. [27] with permission from Elsevier.

Covalent functionalization of the inner lumen

Although it is reported that the hydroxyl groups present in the halloysite inner lumen are thoroughly reactive towards a lot of organic compounds,[69] selective modification of interior remains, so far, a difficult task. Up to now, to the best of our knowledge, only few examples are reported in literature about the covalent modification of inner surface. All of them consider the selective hydrophobization of the halloysite inner surface by reaction with suitable organic compounds. Pioneering work of Lvov *et al.* reported the reaction of the hydroxyl aluminol groups of the inner lumen with octadecyl phosphonic acid without octadeyl phosphonic acid bonding on the siloxane outer tube surface. In this way they obtained a inorganic tubular micelles that shows the typical features of pristine halloysite presenting, at the same time, a hydrophobic core that allows for selective adsorption of hydrophobic molecules such as bisphenol-bis(diphenyl phosphate) or ferrocene.[23, 70] In addition, the hydrophobic modification allows a slower release of the active agents from the lumen with respect to the non-modified halloysite.

A potential new tool for selective modification of silica-aluminum oxides materials is the covalent immobilization of catechol derivative or the reaction with arylboronic acids. In this context it was demonstrated that 2-bromo-N-[2-(3,4-dihydroxyphenyl)ethyl]-isobutyryl amide (Dopa), a catechol derivative, can be covalently linked in aqueous conditions to the alumina innermost surface but not to the silica outermost surface of halloysite.[31]

Arylboronic acid is able to rapidly react with diols in mild conditions and therefore it was used for selective modification of the inner surface of halloysite clay nanotubes.[37] Phenyl boronic acid was found to bind to alumina groups into halloysite lumen and did not bind the external siloxane surface (Figure 10). The tubular hybrid material, with the established Al−O−B linkage, can be used as fluorescence probe for the detection of hydrogen peroxide (H_2O_2) at low levels, which might provide potential applications in the biomedicine field.

H_2O_2 indeed, is involved in several diseases such as diabetes, cardiovascular diseases and cancer, therefore, the development of a system that can efficiently detect H_2O_2 is an important task.

Zhang *et al.*,[43] have developed, following similar procedure reported above, an hybrid system based on halloysite and 1,4 phenylenebisdiboronic acid covalently linked in halloysite lumen; obtaining an efficient system that could be employed as topical preparations to prevent inflammation from occurring. The so-obtained material was used for the immobilization of compressible starch by reaction of the vicinal diol in the carbohydrate and the arylboronic acid units on the halloysite hybrid, forming an hydrogel. The system was used for the loading of pentoxifylline, a model drug, which is released form the carrier in the presence of an inflammation since the hydrogel is disrupted in the presence of H_2O_2 (Figure 11).

Covalent functionalization of the external surface

Besides supramolecular interactions, different strategies for the covalent grafting of functional groups on halloysite surfaces have been widely investigated. Covalent modification of halloysite surfaces overcomes some drawbacks which affect halloysite use. The introduction of organic groups onto the halloysite allows to obtain novel cargo with enhanced performances

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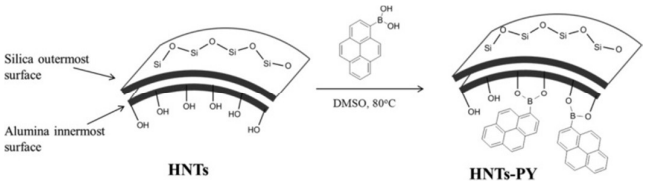

Figure 10. Selective modification of the alumina innermost surface. Reproduced from ref. [37] with permission from American Chemical Society.

for the loading and/or release of molecules and for dispersion of halloysite filler in polymeric matrices.

The siloxane external surface was modified by grafting of organosilane moieties which possesses suitable terminal groups available for further functionalization. Thus, starting from amino, azido, chloro[71] or thiol silanes; they have been obtained several nanomaterials with hierarchal structures.[72, 73]

The silane grafting onto halloysite occurs in several experimental conditions, as refluxing the halloysite powder in the presence of silane in toluene or in solvent free condition under microwave irradiations.

Drug carrier and delivery

The covalent functionalization of the halloysite surfaces is revealed to be a promising strategy for obtaining hybrid systems useful in the drug carrier and delivery field. Indeed, one of the major problems related to the use of pristine halloysite is due to the fact that it shows only weak interactions with guest molecules through hydrogen bonding or van der Waals forces,[74] and fast and non-controlled release.[33]

Thus the introduction of suitable groups on halloysite led a number of advantages. As example, by functionalizing the external surface of halloysite with 3-aminopropyltriethoxysilane (APTES), it is possible to increase the percent loading of ibuprofen (IBU) with respect to pristine halloysite; thanks to the presence in the HNT-APTES nanomaterial of electrostatic attractions, between the aminopropyl groups of the grafted APTES and the carboxyl groups of IBU.[75] in addition, the presence of amino groups slow down the kinetic release of the drug with consequent improvements respect to the pristine halloysite. [33]

Similarly, the so modified nanomaterial was successful used for the immobilization of aspirin molecules.[26] Also in this case, the authors observed an increase in the drug loading with respect to pristine halloysite (from 3.84 to 11.98 wt%, respectively) and an aspirin dissolution rate from the modified halloysite slower than that from pristine halloysite. This strategy was also used for enzyme loading as reported by Kumar-Krishnan *et al.*. The presence of amino groups helped the enzyme glucose oxidase immobilization avoiding its partial deactivation which leads to worse biocatalytic performances. [76, 77]

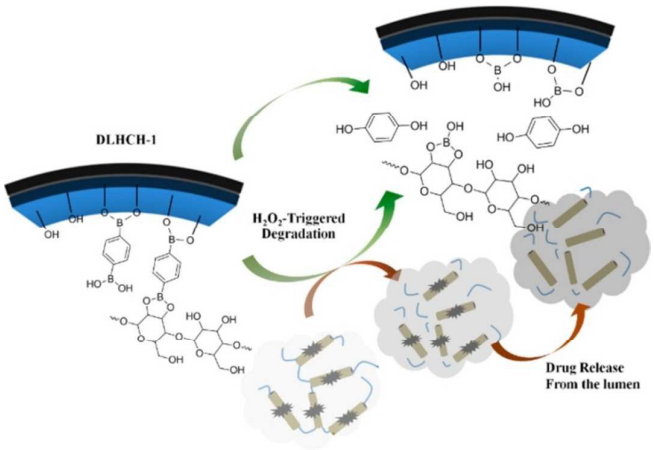

Figure 11. Release mechanism of DLHCH-1. Reproduced from ref. [43] with permission from American Chemical Society.

Amino functionalized halloysite was used as scaffold for the covalent linkage of poly(N-isopropylacrylamide) (PNIPAAM) by condensation of PNIPAAM-COOH to HNT-NH_2 by forming an amide bond.[78] This system was employed for the loading of curcumin and it sustained release triggered by temperature. The *in vitro* results highlighted that the halloysite hybrid can load and release the drug by modulating the temperature and in addition it can deliver curcumin in its active form, preventing its degradation in the gastrointestinal pH conditions. The thermo-responsive behavior of the halloysite modified with PNIPAAM was also exploited for catalytic applications. The functionalized nanomaterials was used for the immobilization of Pd nanoparticles and the catalytic performances were evaluated in the cross-coupling reaction above and below the lower critical solution temperature of the attached polymer (*ca.* 32 °C).[79]

Following a similar procedure both poly(N,N-dimethylaminoethyl methacrylate) and PAMAM (Figure 12 were successful attached on HNT-NH_2 and the obtained systems were used for the loading and release of diphenhydramine hydrochloride, diclofenac sodium, chlorogenic acid, ibuprofen and salicylic acid.[80, 81] The amino functionalized halloysite was also used for the covalent linkage of an antioxidant molecules (Trolox) on the external surface of halloysite (Scheme 1).[82] The novel system possesses several advantages for biological applications. First of all the covalent linkage of antioxidant limits adverse effects such as toxicity by decreasing their steady-state concentration in the system. In addition, thanks to the peculiar halloysite structure, the presence of empty lumen has allowed to load an additional antioxidant (such as quercetin) that acts synergistically with the Trolox presents on the external surface.

In another work starting from the HNT-NH_2 nanomaterial it was possible to obtain a carboxylic acid functionalized halloysite by reaction of the terminal NH_2 group with succinic anhydride. The new scaffold was used for the covalent grafting of chitosan that was used for the loading and release of curcumin and doxorobucin. Owing to the covalent grafting of

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chitosan onto the halloysite, the new drug carrier systems showed hemocompatibility, stability in body liquid and cytocompatibility. In addition both drugs were released in their active form, in a controllable and sustained manner in "tumoral environment" instead of in the normal physiological conditions.[83, 84]

In a recent work,[85] the same authors reported a similar synthetic approach to covalent conjugate on the halloysite external surface, poly ethylene glycol (PEG) for prolonging halloysite circulation time and controlling its dosing interval. Furthermore, to obtain tumor targeting systems, the authors covalent grafted to the PEG functionalized halloysite folate units (Figure 13). Folic acid is a well-known ligand for cancer cells due to the over-expression of the folate receptors on the cellular surface of a wide variety of human tumors while they are not so expressed in normal tissues.

The HNT-PEG-FA hybrid was used for the delivery of doxorobucin (DOX) for the treatment of breast cancer.

The *in vitro* release test highlighted that the DOX is released from the carrier up to 35 h in an acidic environment (pH = 5.3), while it is relatively stable in neutral conditions. Cytotoxicity assays showed that the system can restrain proliferation and induce death of FR^+ MCF-7 cells, while it shows relative low cytotoxicity towards the FR^- L02 cells. Furthermore, the DOX complex can produce more ROS in MCF-7 cells which leads to the apoptosis.

pH sensitive systems were developed by starting from an azido functionalized halloysite (HNT-N_3). This nanomaterial indeed was exploited for the synthesis of triazolium salts based nanomaterial that can stabilize both metal nanoparticles and drugs. In the first case, the halloysite-triazolium salt support was used for the immobilization of palladium nanoparticles and the feasibility of the system as catalyst was investigated in the Suzuki cross-coupling reactions.[86] The use of the triazole in the drug carrier, instead, is a promising strategy to obtain pro-drug systems which could exert synergistic effects with a drug loaded onto the halloysite surfaces. In this context the halloysite based triazolium salts were used as carrier for curcumin and cardanol.[24, 87]

The physico-chemical characterization showed that the electron rich molecules can interact with the positively charged halloysite lumen and besides, with the external surface by $\pi-\pi$ interactions between the aromatic rings of cardanol and curcumin and that of triazolium salts. The coexistence of these two species allows to obtain higher loading efficiency than pristine halloysite and an increased cytotoxicity towards different tumoral cell lines.

The thiol functionalized halloysite was successful used both for the synthesis of the so-called supported ionic liquid like phases (SILLPs)[44, 88] and for obtaining promising multicavity nanomaterials which can load and release two or more drugs in a synergistic and complementary manner.[89]

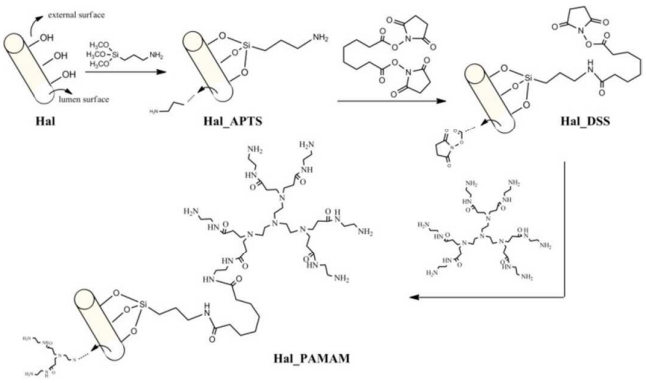
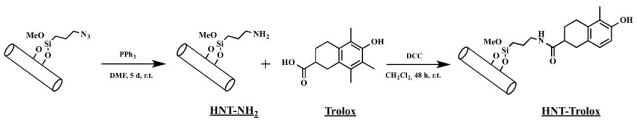


Figure 12. Scheme of synthesis of PAMAM-functionalized halloysite nanotubes. Reproduced from ref. 58 with permission from Elsevier.



Scheme 1. Schematic representation of the synthetic route for the synthesis of the halloysite-Trolox nano-antioxidant.

In the last case it was possible the simultaneous co-delivery of quercetin and silibinin and curcumin and silibinin, since silibinin interacts only with the halloysite lumen, whereas quercetin and curcumin can efficient interact with cyclodextrin cavity.

The *in vitro* release tests performed in physiological conditions by means the dialysis membrane method, showed that in both systems silibinin was better released in acidic solution, therefore in a medium simulating the gastric environment, while the curcumin and quercetin were better released in a medium simulating the intestinal fluid (phosphate buffer pH 7.4). Cytotoxic studies performed towards 8505C cell lines highlighted that the drugs delivered by halloysite hybrids show improved antitumoral efficacy with respect to the free drug. More importantly, if some sugar moieties (such as mannose units, specific for binding cellular lectine) are attached to the cyclodextrin core,[45] it was also observed by fluorescence microscopy, an enhanced cellular internalization due to the carbohydrate-receptor-mediated endocytosis.[90]

By mixing the HNT-SH and cyclodextrin in different proportions it is possible to obtain composite nanomaterials constituted by a network of supramolecular host units joined by means of suitable cross-linkers with a structure similar to a nanosponge (Scheme 2).[91] This hybrid showed swelling ratio of 91.2 ± 1.6% and therefore it is promising for application in the drug carrier field. Based on the above considerations, the HNT-CD system was employed for the loading of clotrimazole in order to develop a system for the treatment of buccal Candidiasis.[92]

Starting from the HNT-SH, it was obtained a prodrug system where curcumin was covalently linked on halloysite surface by a disulphide bond. Furthermore this system showed the presence of an imine bond, sensible to the pH. It was found that the curcumin molecules were released only under exposure of the prodrug to GSH-rich or acidic conditions

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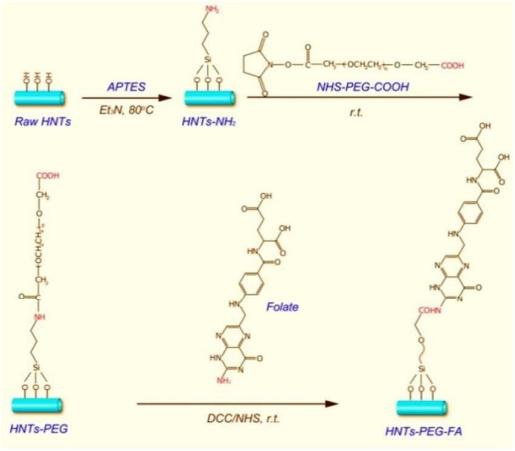


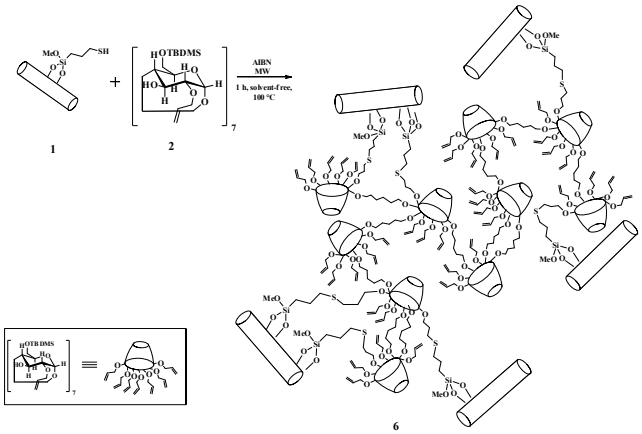
Figure 13. Schematic representation of HNTs-PEG-FA preparation. Reproduced from ref. [85] with permission from American Chemical Society.

similar to that present in hepatocarcinoma cellular microenvironment.[93]

Furthermore, the antiproliferative activity of the prodrug against HA22T/VGH and Hep3B cell lines by vitality count was also assessed. The obtained results showed that the percentages of cells remained survival with a curcumin concentration up to 50 μM were 22% and 16% for Hep3B and HA22T/VGH, respectively (Figure 14), whereas no viability was observed for Hep3B at higher concentration of curcumin (100 μM).

The major problem related to the treatment of malignancies such as tumor is related to the presence, both at the time of diagnosis and at some period during patient lifetime, of metastases. These arise from the so called circulating tumor cells (CTCs) which are the tumor cells that shed into the bloodstream at a very early stage of primary tumor growth. Therefore the development of different strategies to detect the presence of viable CTCs provides a good indicator for the presence of malignant transformations.[94]

In this context, halloysite nanotubes can be efficiently used as capture devices for CTCs.[95, 96] To further improve halloysite efficacy halloysite can be functionalized and after the modified tubes can coated on plastic microtube with or without antibodies.[97]



Scheme 2. Schematic representation of the synthesis of nanosponges hybrid

(compound 6). Reproduced from ref. [91] with permission from American Chemical Society.

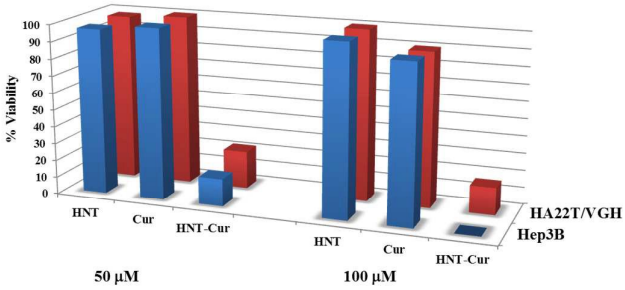


Figure 14. Graphical representation of cell viability of HA22T/VGH and Hep3B in presence of curcumin, halloysite and HNT-Cur prodrug. Relative errors are in a range of ±0.5–1% (results correspond to the mean ± standard error mean of three independent assays with three replicas each). Reproduced from ref. [93] with permission from Elsevier.

Liu *et al.*, reported the preparation of patterned halloysite coatings on flat glass substrate via drying the halloysite aqueous dispersion in a slit-like confined space (Figure 15).[96] Once formed, the authors investigated the ability of the systems to capture several tumor cell lines sincluding MCF-7, HepG2, Neuro-2a, A549, and B16F10. They found that greater is the surface roughness of the halloysite coating, greater is the capture efficacy- To further increase the capture efficacy of halloysite coating, anti-EpCAM was conjugated on the surface of halloysite. The antibody conjugated halloysite surface showed 92% of capture efficacy towards MCF-7 at 3 h.

The same authors proposed another strategy to produce uniform halloysite coating with large areas by a simple thermal spraying method.[95] since it was found that a surface more rough both promote the cell capture and stimulate tumor cells to produce a large amount of extracellular matrix, also in this study the authors conjugated the halloysite coating with anti-EpCAM. This new system can also specifically capture rare tumor cells from peripheral blood samples and blood samples of patients with metastatic breast cancer. Furthermore, in this study, the coated halloysite nanotubes were used for the immobilization of doxorobucin, in order to obtain a carrier system which can efficiently kill tumor cells.

Hydrogels

Fan *et al.* reported the preparation of halloysite-sodium alginate/hydroxyapatite nanocomposite beads by generation of hydroxyapatite (HA) in a nanosized regime. The beads were used for load and release of diclofenac sodium. The combination of halloysite with a tubular structure and HA nanoparticles could limit the flexibility of the alginate polymer chains, which is the principal reason for the enhanced loading of the active pharmaceutical component. Moreover, a sustained release behavior is achieved.[98]

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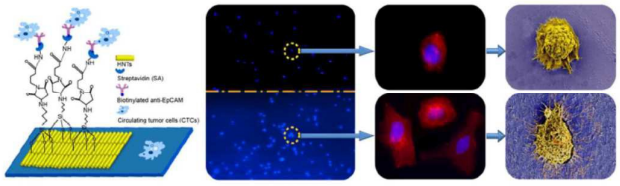


Figure 15. Polystyrene sulfonate sodium modified halloysite nanotubes for tumore cell capture. Reproduced from Ref. [96] with permission from The Royal Society of Chemistry.

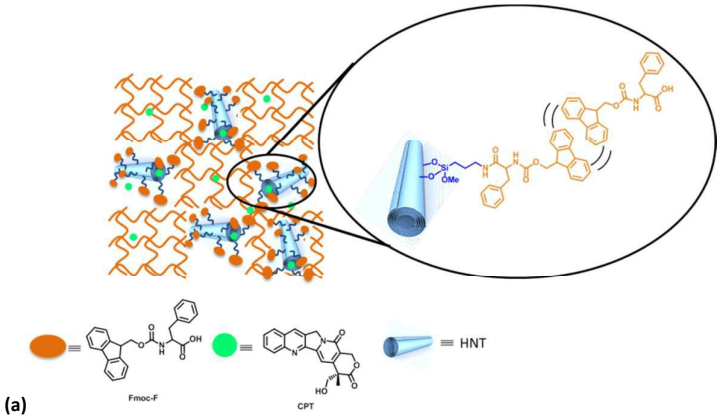
Magnetic microspheres, 2-hydroxypropyltrimethyl ammonium chloride chitosan/Fe_3O_4/halloysite nanotubes/ofloxacin (HACC/Fe_3O_4/HNTs/OFL), for the controlled release of OFL were synthesized by *in situ* crosslinking with glutaraldehyde in the spray-drying process. The magnetic microsphere were employed as carrier for ofloxacin. It was found that halloysite have remarkable effect on the roughness of the surface, which decreases the entrapment efficiency and accelerates the release of OFL though an increase in halloysite content is conducive to the adsorption of OFL into halloysite. However, the introduction of halloysite can improve the bioavailability of OFL in the gastro-retentive drug delivery system.[99]

Due to its features, among them hemostatic and anti-infectional activities, chitosan can be also used as wound healing. Chitosan can accelerate the infiltration of polymorphonuclear cells at the early stage of wound healing, followed by the fabrication of collagen by fibroblasts. The addition of halloysite aids in faster re-epithelialization and collagen deposition. These properties are attributed to the particular characteristics of halloysite and their combination with chitosan. The obtained results show that these advanced halloysite-Chitosan bionanocomposite sponges have many potential applications for diabetic, burn and chronic wound infections.[100]

Controlled loading and sustained release of 5-aminosalycilic acid into halloysite was proved.[101] The introduction of 5-aminosalycilic acid/halloysite within the thermoplastic starch generates a biopolymer that can transport the drug through the acidic medium of the stomach and that can release it in sustained manner in the colon. The development of a halloysite-Starch bionanocomposite thus should be considered as a basis for further development of colon specific drug delivery formulations using halloysite nanotubes.[102]

The modification of halloysite external surface with amino acid derivatives allows to obtain nanohybrids that can be incorporated in a peptide hydrogel to obtain novel drug carrier. For this purpose, the HNT-NH_2 nanomaterial was used as intermediate to covalent link Fmoc-phenylalanine (f-HNT) (Figure 16).[103] This amino acid is able to give hydrogels of high density featured by the presence of uniform entangled fibers

The presence of the amino acidic functionality on halloysiteT surface allows specific π-π interaction between modified halloysite and Fmoc-phenylalanine (Fmoc-Phe) generating hybrid supramolecular hydrogel with affected physico-chemical properties. Deep investigation of hybrid gel properties, indeed, showed that the introduction of an halloysite filler in the hydrogel matrix induced decrease in thermal stability but, in general, a concomitant increase in the gel strength. The hybrid hydrogel was employed for camptothecin delivery. Release tests in physiological conditions showed a synergistic action of halloysite and gel matrix, as accounted for by the best drug dispersion due to the presence of the filler and the slower rate of release as a consequence of the halloysite incorporation in the gel phase.



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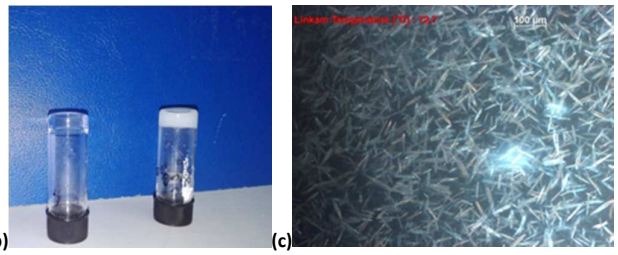

Figure 16. (a) Schematic illustration of Fmoc-Phe/f-HNT/camptothecin hybrid gel; (b) on the left, transparent hydrogel, on the right, hybrid hydrogel containing 0.1% of f-HNT; (c) POM images of Fmoc-Phe/f-HNT/camptothecin at 72.7 °C. Adapted from ref. [103] with permission from Royal Society of Chemistry.

Liu *et al.*,[104] have incorporated halloysite into cellulose NaOH/urea solution to obtain composite hydrogels using epichlorhydrine as crosslinking agent. The introduction of the inorganic filler in the cellulose matrix led to an increase in the physico-chemical properties of the final nanocomposite with respect to the pristine cellulose hydrogel. The so-obtained hydrogel was used as carrier for curcumin and by cytotoxicity assays, the authors found that the nanocomposite possesses a good biocompatibility towards MC3T3-E1 and MCF-7 cells; whereas after the loading of biological molecule it was observed a strong inhibition of cell viability.

The HNT-NH_2 was also used for the grafting of poly(methyl vinyl ether-*co*-maleic acid onto halloysite external surface.[105] This polymer was chosen since it possesses appealing muchoadesive properties which can promote drug adsorption. The hybrid was used for curcumin inclusion and the resulting complex was encapsulated into a pH responsive polymer, such as the MF grade of hydroxypropyl methyl cellulose (dissolution at pH> 6.0). Following this approach the curcumin molecules can withstand the harsh conditions of the gastrointestinal tract and they can be released only in the small intestine.

Tissue engineering and gene delivery

Halloysite nanotubes are promising materials for tissue engineering since they meet the specific requirements of for a good scaffold, as example it is mechanically strong, porous and biocompatible. In this context Zhou *et al.*[106] prepared a halloysite-Chitosan scaffold for tissue engineering application, via solution mixing and freeze-drying method. The chitosan–halloysite scaffold has shown enhanced compressive strength, compressive modulus, and thermal stability compared with the pure chitosan scaffold. More importantly, cytotoxicity results showed these materials do not exert any cytotoxic effect towards NIH3T3 cells. Cell morphology results showed that cells can be attached and developed well on all of the materials.

Thanks to the high performance and biocompatibility, halloysite-Chitosan as well as halloysite-Alginate bionanocomposites have also possible applications in bone tissue engineering. In order to verify cell attachment and viability on the bionanocomposites several studies were carried out. The presence of halloysite dispersed in the polymeric matrices increase the roughness of the bionanocomposite and therefore them showed an increase in the cell attachment and growth.[106, 107]

Halloysite functionalized with hydroxyapatite were successful dispersed in polymeric matrices, obtaining novel nanomaterial with enhanced cell attachment and cell viability. The introduction of halloysite promotes the generation of new reactive sites where the proteins can interact by electrostatic forces and hydrogen bonds. In addition, a polymeric matrix reinforced with halloysite shows a surface nanoroughness that acts as an anchoring framework.

Fakhrullin *et al.*,[108] have employed the freeze-dry technology for fabrication of porous biopolymer hydrogels, based on chitosan, agarose and gelatin, doped with halloysite nanotubes to obtain biocompatible and biodegradable scaffolds.the authors have demonstrated that the addition of 3–6 wt% of halloysite nanotubes leads to a significant improvement of mechanical stability and wettability of the biopolymer scaffolds without harming the cell growth *in vitro*. By means of studies on the cell distribution and morphology it was highlighted that cells can be efficiently attached and grown well on halloysite-scaffolds. The *in* study on rats have shown a good biocompatibility of the so-obtained scaffold with a slight inflammatory effect without rejection of implants and with a 6 week period of degradation, as confirmed by histological examination. Finally, the blood perfusion in the implanted area was estimated and it was observed the full restoration of blood supply in six weeks.

Another approach to treat diseases, especially the genetic ones, is the delivery of nucleic acids directly into the patient's cells. After delivery, the gene can be replicated and/or expressed. Recently, the research of an appropriate vector for the gene delivery has been focused on inorganic nanomaterials, which show good biosafety, easy preparation and immunogenic characteristics. In this context, Liu *et al.*,[109] grafted PEI on halloysite to develop vector for the loading and intracellular delivery of DNA. Since long tubes could lead to cell injury and inflammation, the authors firstly treated halloysite by ultrasounds in order to shorter them and then, chemically grafted PEI onto halloysite external surface (HNT-PEI).

The HNT-PEI hybrids were used for delivery of pDNA. The biological assays on the hybrid complex showed that it possesses low toxicity and reasonable blood compatibility. In addition, the *in vitro* transfection results highlighted high transfection efficiency towards 293T and HeLa cells and therefore, the HNT-PEI/pDNA could be promising for application in gene therapy towards many diseases such as

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cancer. A similar approach, where the external surface of halloysite was functionalized by means of electrostatic layer-by-layer of positively charged PEI (f-HNT) was also exploited,[110] for the delivery of siRNA into cancer cells and noninvasively image the process simultaneously. To reach this goal, Jia *et al.*, covalently conjugated with siRNA, CdSe QDs to obtain a fluorescent label probes.

The developed f-HNTs carriers exhibited efficient intracellular transporting and high delivery efficiency of siRNA. Furthermore, the f-HNTs-mediated siRNA could effectively induce the knockdown of the target surviving gene in PANC-1 cells and improve the antitumor activity of siRNA.

γ-aminopropyltriethoxysilane functionalized halloysite (f-HNT) were used for the delivery of antisense oligodeoxynucleotides (ASODNs), as a therapeutic gene for targeting survivin.[111] All characterizations performed, highlighted that the f-HNT/ASODNs complexes could efficiently improve intracellular delivery and enhance antitumor activity of the nucleotide.

Environmental

Due to their sustainability, halloysite nanotubes were recently proposed for specific environmental applications, such decontamination and filling of bioplastics.

Within the decontamination purposes, halloysite is a suitable adsorbent material for both inorganic and organic pollutants because of the large specific area and tunable surface properties.[112] The modification of halloysite surfaces through supramolecular interactions generated nanotubes with enhanced adsorption capacity towards specific contaminants including aliphatic and aromatic hydrocarbons.[21, 51] The functionalization of halloysite inner surface with sodium alkanoates induced the formation of nanotubes with a hydrophobic cavity, which exhibited a relevant affinity towards apolar molecules.[19, 21] The sodium alkanoates/halloysite hybrids can be considered as inorganic tubular micelles being that their external surface possesses a hydrophilic nature, while their cavity is hydrophobic.[19, 21] It should be noted that the hydrophobicity degree of the lumen depends on the specific surfactant organization.[58] It was observed that the presence of both sodium dodecanoate and sodium tetradecanoate confers a stronger hydrophobic character as a consequence of the formation of complex surfactant structures inside the halloysite lumen, while a smaller hydrophobization effect was detected for sodium undecanoate/halloysite composite, which presents a monolayer surfactant adsorption on the halloysite internal surface.[21] Accordingly, the removal ability of decane from both liquid and vapor phase was higher for nanotubes modified by sodium alkanotes with a longer alkyl chain.[21] As evidenced by surface tension measurements over time (Figure 17a), both pure halloysite and sodium undeacanote/halloysite hybrid are not able to remove the liquid decane in contact with the aqueous phase being that the surface tension is constantly at ca. 50 mN m^{-1}. On the other hand, the presence of nanotubes modified with sodium tetradecanoate induced a jump of the surface tension (from ca. 50 to 72 mN m^{-1}, which are the values for decane and water, respectively) highlighting the removal of the hydrocarbon at the interface (Figure 17a). The removal process of decane at the interface with water is sketched in Figure 17a.

Fluorescence experiments (Figure 17b) were performed to investigate the adsorption capacity of anionic surfactant/halloysite hybrids towards Nile Red, which is very sensitive to the hydrophobicity of the microenvironment. The fitting of the fluorescence data allowed to determine the distribution constant (K_N) of Nile Red between the water and the hydrophobic lumen of nanotubes modified with sodium dodecanoate and sodium dodecylsulphate.[58] The different K_N values (40 ± 12 and 4.2 ± 1.8 dm^3 g^{-1} for sodium dodecanoate/halloysite and sodium dodecylsulphate/halloysite, respectively) are consistent with the different surfactant organization evidenced by SANS data analysis.[58] Ecocompatible nanosponges for adsorption of oils were fabricated by functionalization of halloysite surfaces with cucurbit[8]uril (CB[8]) molecules.[51]

The amino functionalized halloysite was used for the efficient removal of Pb^{2+} from polluted waters. Also in this case, the f-HNT showed a considerable higher adsorption ability towards Pb^{2+} than that of pristine halloysite.[113]

It was observed that the modification with CB[8] significantly enhances the adsorption capacity of the nanotubes towards toluene (in vapor phase) and pyrene (in aqueous phase).[51] The halloysite modification with hexadecyltrimethylammonium bromide produced nanotubes with excellent removal capacity of Cr(VI) from waste waters.[114] The adsorption capacity of the modified nanotubes depends on the pH conditions of the aqueous media. Halloysite was successfully used for efficient water filtration as demonstrated in adsorption studies using cationic Rhodamine 6G and anionic Chrome azurol S as cationic and

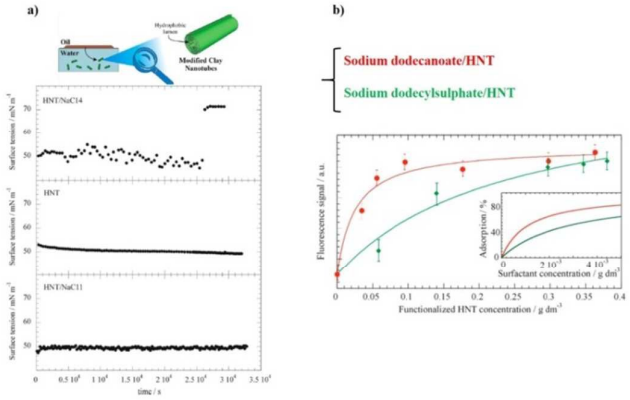

Figure 17. a) Surface tension as a function of time for aqueous halloysite, halloysite/sodium undecanoate (NaC11), and halloysite/ sodium tetradecanoate (NaC14) dispersions in the presence of a decane at the interface. Adapted from ref. [21] with permission from American Chemical Society. b) Integrated fluorescence signal for Nile Red (10^{-8} mol dm^{-3}) as a function of the functionalized halloysite concentration. Inset shows the percent of Nile Red adsorbed by the functionalized halloysite as a function of the surfactant concentration. Adapted from ref. [58] with permission from American Chemical Society.

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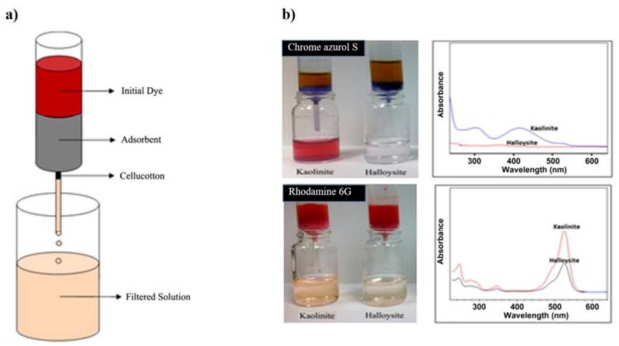

Figure 18. a) Sketch of the water filter experiment. b) Demonstration of the water filters prepared by 1 g of halloysite and kaolinite clays, tested on Chrome azurol S and Rhodamine 6G solutions with 300 mg dm^{-3} concentration. UV spectra of the filtered water samples are demonstrated on right for clear presentation of the unfiltered dye. Adapted from ref. [115] with permission from Elsevier.

anionic dyes, respectively.[115] The filtration experiment is sketched in Figure 18a. Compared to kaolinite, halloysite exhibited a higher removal capacity towards both dyes (Figure 18b) due to its nonporous structure.

In regards to the development of composite bioplastics, halloysite nanotubes were used as fillers for biopolymeric matrices, such as cellulose ethers,[41, 116] chitosan,[106, 107, 117] polylactic acid,[118-120] alginate[41] and pectins.[121 122, 123]As evidenced in a recent review,[124] the physico-chemical properties of the biopolymer/halloysite nanocomposites are competitive to those of the traditional plastics. Accordingly, the potential applications of the biopolymer/halloysite nanocomposites are relevant in several technological applications as alternatives to the petrochemical-based materials.[125-132] The uniform distribution of the nanotubes into the matrix generated nanocomposite materials with improved mechanical and thermal properties with respect to those of the pristine polymers. Specifically, the increase of the polymer thermal stability was attributed to the barrier effect of the filler as well as to the encapsulation of the polymeric chains within the halloysite cavity.[41] Literature reports that the peculiar mesoscopic structure affects the wettability of the bionanocomposites. On this basis, biofilms with tunable surface properties can be obtained by changing the biopolymer/halloysite composition of the nanocomposites.[116] As evidenced by the initial water contact angle data (Figure 19), pectin/halloysite nanocomposites (filler content = 60 wt%) showed an enhancement of the surface hydrophilicity respect to that of the pure biopolymer because of the uniform distribution of the nanotubes. On the other hand, the addition of halloysite into hydroxylpropylcellulose generated nanocomposites with a sandwich-like structure and a consequent enhancement of the surface hydrophobicity (Figure 19).[116]

Furthermore, it was proved[119] that filling biopolymers with halloysite allows to fabricate biofilms with improved vapor barrier properties, which can be useful for specific technological applications including the food packaging.

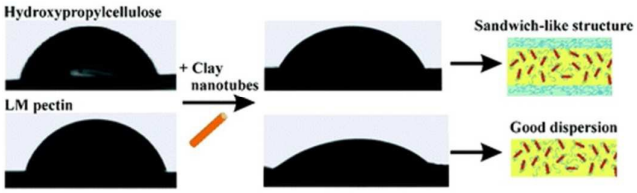

Figure 19. Images of the water contact angle just after the deposition on the surface of pure biopolymers and their corresponding biopolymer/halloysite nanocomposites (filler concentration of 60 wt%). The mesocopic structure of the composite biofilms are sketched. Adapted from ref. [116] with permission from American Chemical Society.

Chitosan/halloysite composite films were successfully employed for the entrapment of horseradish peroxidase (HRP) and adhesives for the electrode surfaces. Consequently, these biofilms could act as catalytic platform for the reduction of H_2O_2.[133] The immobilization of HRP into the chitosan/halloysite hybrid through cross-linking by glutaraldehyde revealed promising in wastewater treatment.[134]

Halloysite nanotubes modified by a click reaction involving azides and alkynes allowed to obtain well-defined microfibers that were dispersed in chitosan and hydroxypropyl cellulose matrices[117]. These bionanocomposites showed an increase of the elastic modulus with respect to both pure biopolymers and hybrid films reinforced with pristine halloysite. Functional nanocomposite materials were prepared by filling polymeric matrices with halloysite loaded with chemically and biologically active compounds, such as biocides,[50, 135] antioxidant[50] and anticorrosion molecules.[136] As example, pectin was filled with cucurbit[6]uril (CB[6])/halloysite hybrid containing essential peppermint oil (PO) by means of the aqueous casting method sketched in Figure 20a.[50] The presence of the essential oil conferred antioxidant activity to the composite biofilm evidenced by the large inhibition percentage (41%) of the free radical DPPH.[50] In addition, the biocomposite film loaded with the peppermint oil was efficient in antimicrobial activity against *Escherichia Coli* and *Staphilococcus Aureus* strains (Figure 20). Specifically, it was observed that the temperature increase favors the antibacterial capacity (Figure 20b).

Gorrasi et al[121] obtained composite films with specific functionalities by filling pectin with halloysite containing rosemary essential oil, which could be used for potential applications in packaging field. Recently, the addition of halloysite loaded with salicylic acid into pectin matrix allowed to fabricate biofilms with excellent thermo-mechanical properties as well as effective antimicrobial activity against several bacteria including *Salmonella*, *Pseudomonas Aeruginosa*, *Escherichia Coli* and *Staphilococcus Aureus*.[135] A novel composite film was prepared by the combination of low-density polyethylene (LDPE) and halloysite containing carvacrol and thymol inside its lumen.[137] The formation of an inclusion complex between carvacrol and thymol induced a thermal stabilization effect on both essential oils. Interestingly, a synergetic antimicrobial actions of carvacrol and thymol was detected by biological tests conducted on *Escherichia Coli*.

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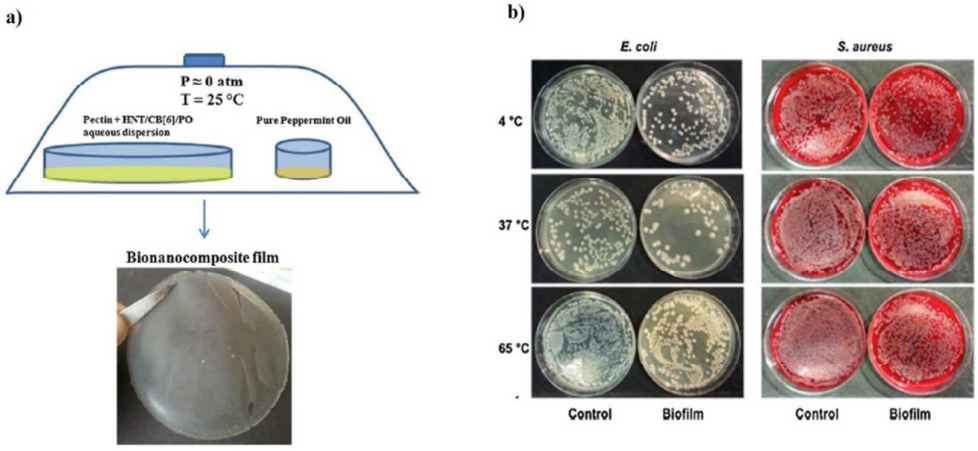

Figure 20. a) Schematic representation of the apparatus for the preparation of the composite biofilm based on pectin and CB[6]/ halloysite hybrid containing essential peppermint oil (PO). b) Antibacterial activity of pectin/halloysite/PO films against E. coli and S. aureus at 4, 37 and 65◦C incubated for 30 min. Adapted from Ref. [50] with permission from Elsevier.

Conclusions

Halloysite nanotubes have been attracted great interest since they are biocompatible materials with appealing properties. The functionalization of both halloysite surfaces, by means of supramolecular interactions or covalent modifications, opens up different ways to obtain interesting nanomaterials which show improved biological properties with respect to pristine halloysite. In this context, beside the functionalization, the possibility to load and slowly release from the halloysite lumen, molecules with biological properties, allows to synthesize promising drug carrier and delivery, stimuli-responsive systems and so on. Recently, thanks to the halloysite properties, several systems for tissue regeneration and gene delivery have been also investigated. Furthermore, the capacity to load molecules was also exploited for environmental purposes as the removal of pollutant or for the release of biological active molecules for active food packaging.

Conflicts of interest

"There are no conflicts to declare".

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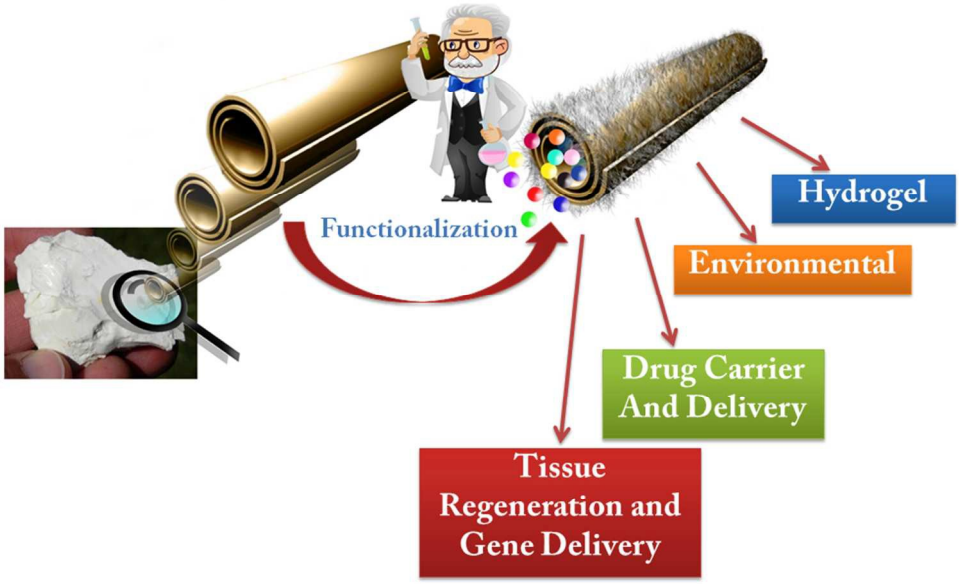

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