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Biomedical potential of clay nanotube formulations and their toxicity assessment

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Abstract

Introduction: Halloysite clay nanotubes (HNTs) are a naturally abundant and biocompatible aluminosilicate material with a structure able to encapsulate 10-20 % of drugs. These features are attractive towards the clinical application in controlled drug delivery, tissue engineering and regenerative medicine.

Areas covered: We describe the application of HNTs as a viable method for clinical purposes, particularly developing formulations for prophylaxis, diagnosis and therapeutics, having a special attention to these nanotubes bio-safety. HNTs may be used for pharmaceuticals, biopharmaceuticals, wound healing, bone regeneration, dental repair, hair surface engineering and biomimetic applications.

Expert opinion: HNTs are a versatile, safe and biocompatible nanomaterial used for drug encapsulation for numerous clinical applications. The studies here reviewed confirm the HNTs biocompatibility, describing their low toxicity. Further developments will be made regarding the long-term efficacy of halloysite-based treatments in humans, concentrating mostly on topical applications.

Keywords: Halloysite clay nanotubes; Drug therapy; Biosensor; Bioimaging; Tissue engineering; Regenerative medicine; Toxicity.

Article highlights

- Drug loading in halloysite clay nanotubes (HNTs) for distinct medical therapies.
- Enzyme and DNA immobilization in HNTs for diagnosis and therapy.
- Composites of HNTs as potential theranostic agents.
- Drug loading in HNTs for wound-healing and bone/dental repair.
- HNTs for hair coating with targeted drug delivery towards parasitic infection treatment.
- HNTs are a safe, biocompatible and abundantly available nanomaterial.

1. Introduction

The development of medicine calls for the use of nanotubes particularly regarding prophylaxis, diagnosis and therapeutic strategies. Synthetic materials are usually associated with hazardous and toxicity concerns. A search for natural green materials, among which are nanoclays, is very promising. Clay nanotubes like halloysite are abundantly available in nature and have been used for centuries in household and clinical applications as safe bulk material without exploiting its nanoscale features. Clay nanotube encapsulation protects drugs from degradation and extends drug release patterns [1].

Halloysite clay nanotubes (HNTs) are inexpensive, biocompatible, and highly functionalize nanomaterials. They are formed by rolling flat sheets of kaolinite (aluminosilicate) 15-20 times [1], which gives rise to a unique hollow tubular structure with a lumen diameter of 15−20 nm, an outer diameter of 50-70 nm and a length of 400-800 nm [2]. **Figure 1(B-E)** illustrates the shape and size of HNTs with scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) imaging. The tube has $Al_2Si_2O_5(OH)_4 \cdot 2H_2O$ [3] composition.

HNTs inter layer wall periodicity is 0.72 nm, where Al is exposed in a gibbsite octahedral sheet (Al−OH) at the inner surface and siloxane groups (Si−O−Si) are presented at the external surface [4,5]. This configuration confers a positively charged inner lumen, composed mostly of aluminum hydroxide and a negatively charged surface, constituted of silicon dioxide. The colloidal features of HNTs are comparable to 100 nm diameter silica nanoparticles. HNTs are easily dispersed in water, acetone, alcohol and polar polymers. Having a negative zeta potential *ca.* −30 mV in a wide pH interval (3 to 8), HNTs form suspensions stable for 2-3 h [6]. Different inner/outer charges of these clay nanotubes allow for selective immobilization of charged molecules (drugs, proteins, DNA) inside or outside. The capture of molecules to the outer surface and internal lumens of HNTs is driven by electrostatic adsorption. The most interesting for long lasting drug release is the tubes' lumen loading or, intercalation between the wall multilayers. The HNTs lumen is rather large (10-20 vol %) and enables the loading of different sized molecules, including proteins and DNA. The drug loading process, particularly of negatively charged drugs, neutralizes the lumen charge and enhances the zeta potential of HNTs to *ca.* −60 mV, resulting in colloid formulations stable for months. The HNTs drug release profiles can take several hours to days, but those may be further modified using silanization, the tube dextran coating or the end stoppers to achieve superior drug release profiles. Intracellular drug delivery was achieved with enzyme-activated release triggering, using the tube's dextran coating. This brings relevance for anticancer targeted therapy due to advantages shown using the tubes elongated shape in cell internalization which is advantageous over spherical shape particles [1,2,7].

In cooperation with the National Institute of Health, our ability to reproduce results consistently with naturally supplied clay nanotubes was questioned. It is not seen as a problem since within single supply, all physicochemical characteristics of the halloysite nanotubes will be identical. Such large amounts of the material enable R&D as well as a full-scale pharmaceutical production. The following multi-ton supply may have minor differences which will require only slight adjustments in the production process. A company can purchase up to 100 tons of halloysite from at least four commercial sources in US, UK, China, and Turkey.

HNTs were first used as a carrier material for the sustained delivery of drugs and biological active substances by Price and Lvov [3]. Several experiments have been followed focusing the encapsulation and modified controlled release of substances of clinical interest: drugs, such as dexamethasone, furosemide, nifedipine [4] and resveratrol [8]; biopharmaceutical agents such as enzymes (laccase, glucose oxidase and lipase [2]) and therapeutic nucleic acids; natural compounds, like vitamins [9]; imaging contrasting agents and biosensors [1]. Additionally, HNTs have been a used for tissue engineering and regenerative medicine by the development of tissue scaffolds and self-healing nanocomposites targeted for wound healing, bone cement, dental cement, as well as regarding the reinforcement of microvascular networks, and hair treatments by a coating process [1,10-12]. HNTs have been employed for the oral and topical administration of drugs [13,14]. Orally, they have already been proven to work as an efficient compression excipient in tablets and capsules formulation [15].

Several toxicity studies have been addressed recently, demonstrating the feasibility and high biocompatibility of this nanomaterial. However, the topical route remains the most promising field of application by HNTs being highly biocompatible but not biodegradable [16]. In this review, we emphasize the applications of HNTs in the clinical field and the formulation of new drug delivery systems.

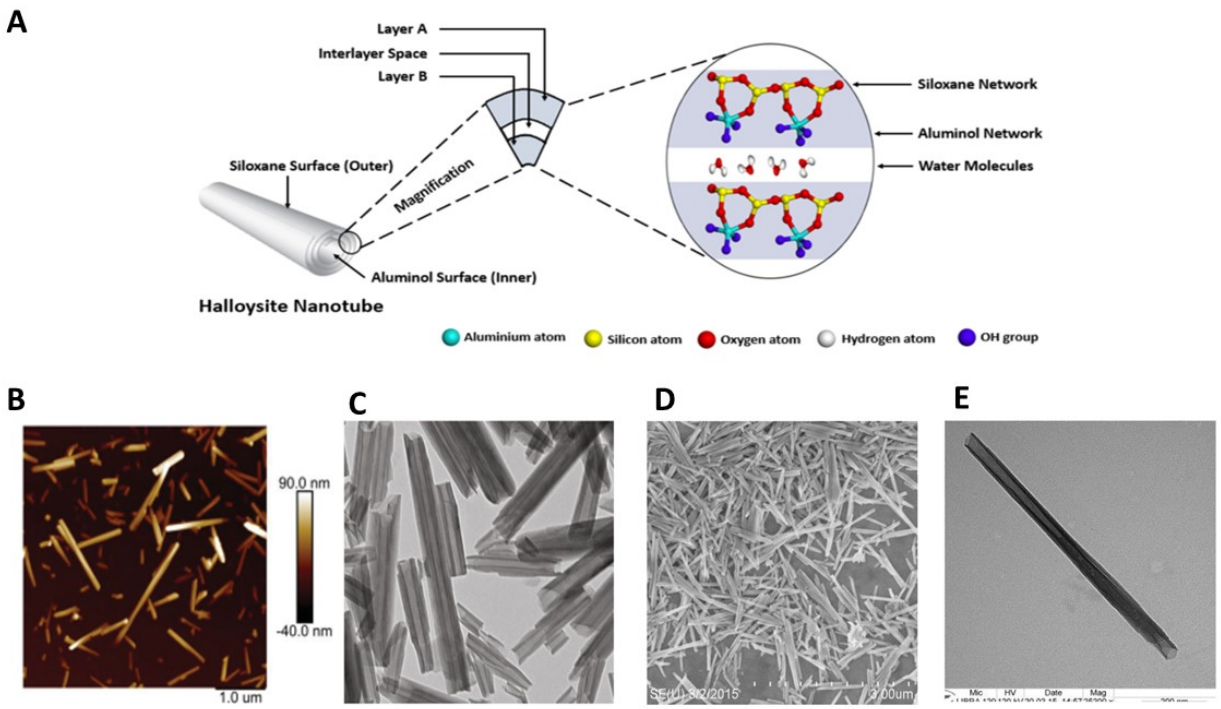

Figure 1. Schematic representation of the clay nanotubes (A). AFM (B), TEM (C) and SEM (D, E) images of HNTs (bottom). (A) reprinted with permission from [17] Copyright 2018, Elsevier; and (B, C, D, E) Reprinted with permission from [18] Copyright 2016 Wiley Online Library.

2. Loading and release strategies

The clay nanotubes enable drug encapsulation by three main mechanisms, including lumen loading assisted by a vacuum method, physical electrostatic adsorption, inside or outside, and intercalation. The improvement of drug loading/release patterns requires a surface modification and formation of caps at the end of the nanotubes.

Lumen loading may be achieved due to the hydrophilicity of the lumen. This enables the high capillary pressure in polar solvents (up to 200 atm) to move aqueous drug solutions into the tubes [4,6]. In this procedure, HNTs are mixed with close to saturated drug solutions. The suspension is placed to a vacuum jar, which triggers the replacement of the air present inside the sample by the drug. Drug-loaded-HNTs are then separated by centrifugation and dried at 60–70 °C in an oven. This process is repeated 3 times to assure maximum loading. Typically HNTs loading with drug is

7-9 wt. % [1,3]. Recently, the mechanism beyond this procedure has been explained by thermodynamic evidence. Thanks to the Gibbs-Thomson effect, the confined water fraction contained in the halloysite exhibits a faster evaporation rate, leading to the drug molecules precipitation into the lumen. Such occurrence is favored by vacuum pumping [19]. **Figure 2(A)** illustrates the encasing of paclitaxel into HNTs from an acetone solution by the lumen loading procedure [15]. This simple method allows a drug release profile of 10-20 h [4,15].

Furthermore, the alumina-based lumen of pristine HNTs of 10 vol. % may be further increased to 20-30 vol. % by etching, *i.e.*, with acid treatment for several hours. This treatment triples the drug loading capacity, from *ca.* 10 up to 20-30 wt. %, as a consequence of the lumen diameter enlargement from 15 to 25 nm, and, naturally, with a parallel enhancement of the tubes' surface area from *ca.* 60 m^2/g to 250 m^2/g [1,20]. In addition, the lumen may be submitted to a hydrophobization procedure to encapsulate low water-soluble drugs from organic solvents. This can be obtained following the adsorption of anionic amphiphilic molecules in the positively charged lumen, conferring the innermost hydrophobic properties without altering the negative charge of the tube's surface. A kind of clay micelle is thereby obtained, preserving nanotubes water dispersibility together with an inner hydrophobic core, that may encapsulate low water-soluble anticancer drugs [21].

The characteristic dual surface chemistry of HNTs, particularly the positively charged alumina lumen together with the negatively charged external silica-based layer allows for selective drug loading. This occurs by the physical adsorption mechanism of negatively charged molecules in the positive lumen as well as positively charged molecules onto the negative surface. Many drugs have been encapsulated through this procedure, including negatively charged DNA and proteins in aqueous media at pH value above their isoelectric point (pI) [2,18].

The bonds between the drug and the HNTs facilitate biofunctionalization, and enhance the stability of the nanotube nanoformulations [22]. Conjugation of polymeric materials on the surface of the nanotubes, can be carried out. An example of a modification agent is 3-aminopropyltriethoxysilane (APTES), which converts the negatively charged surface of HNTs into positively charged, owing to the amine groups allowing the bonding of biomolecules [22]. This modification also contributes for extending the drug release profile [9,23-26]. Copper–benzotriazole film coatings

at the ends of the nanotubes have been applied to prolong the drug release pattern for 20-100 h [6]. **Figure 3(A)** illustrates brilliant green loading using copper–benzotriazole HNTs coatings. Tetraethoxysilane (TEOS) and octyltriethoxysilane (OTES) have been used to modify the wettability and the drug release profile of the nanocomposites [27]. Layer-by-Layer (LbL) self-assembly on the HNTs surface is made by the alternate adsorption of oppositely charged polyanions. The deposition of highly active coatings has extended drug release, resulting in superior therapeutic performances [28-30].

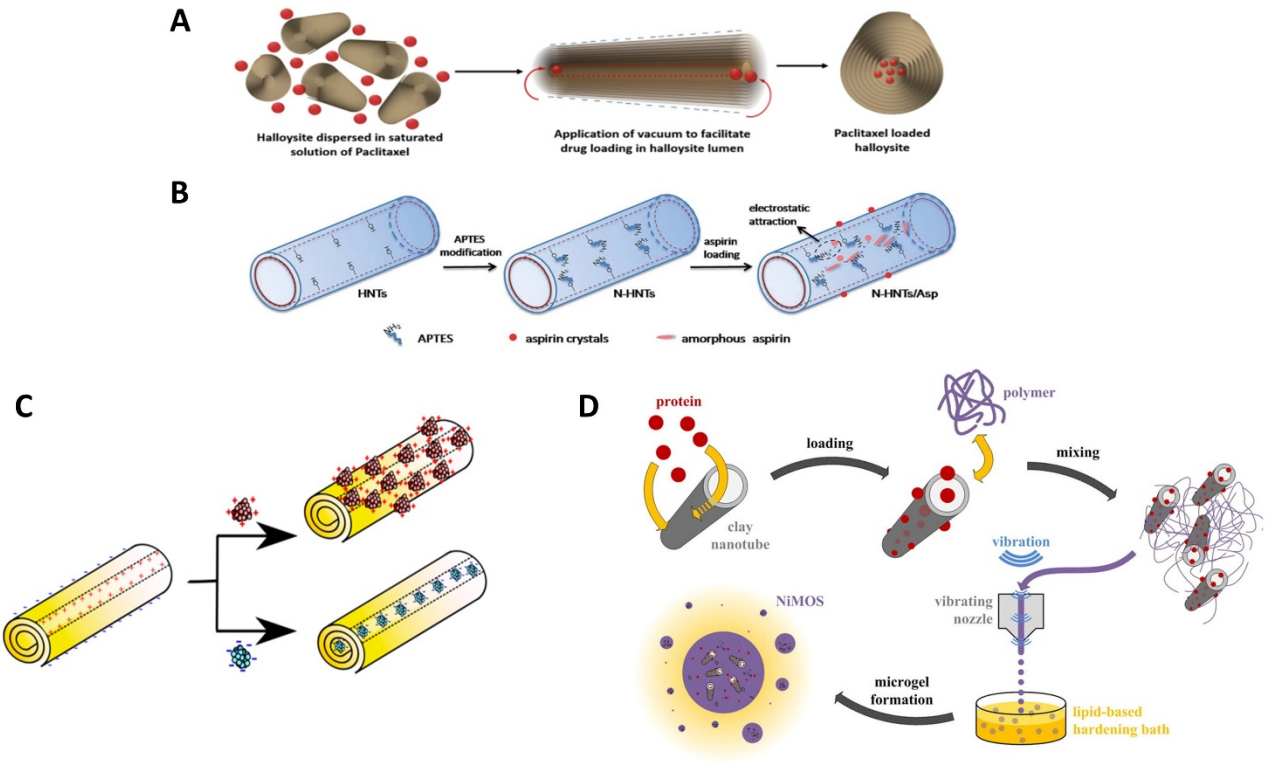

Figure 2. Drug (paclitaxel) lumen loading with vacuum method (A); aspirin physical adsorption on the surface of APTES modified HNTs; (B); representation of positively and negatively charged enzymes immobilization on the surface and in the lumen of HNTs (C); HNTs-in-microgel oral systems for protein delivery production (embedded in a microgel) (D). (A) reprinted with permission from [15] Copyright 2017, Elsevier; (B) reprinted with permission from [23] Copyright 2014, The Royal Society of

Chemistry (C) reprinted with permission from [2] Copyright 2015, American Chemical Society; and (D) reprinted with permission from [13] Copyright 2016, Elsevier.

Additional modifications of the surface have been carried out for drug targeting through cellular recognition. HNTs have been modified by dextrin coating. **Figure 5(A4)** images a dextrin cap at the end of the nanotube. It is enzyme-responsive and targets drug release to the intracellular medium through the enzymatic degradation of dextrin with glycosyl hydrolases [7]. A folic acid was also used in order to target the drug release to over-expressed folate-receptor cells [31,32] and biotin [33]. A redox-responsive target capability was also allowed by the presence of a disulphide bond between thiol groups and per-thiol-β-cyclodextrin (β-CD-$(SH)_7$), sensible to glutathione present in high concentration in cancer cells [31]. Other targeting strategies sensible to the glutathione is the linkage of cysteamine by a disulfide bond to the external surface of HNTs [34]. Drug-loaded HNTs may be embedded into bulk polymers [3], gels (as nanoparticles-in-microgel oral systems (NiMOS)) [13,35] and electrospun microfibers [25,36,37], which contribute to the stability enhancement of the system and attribute much longer drug release (up to two weeks).

The tubes' wall intercalation procedure is less common when small drug molecules are loaded into the interlayer space of HNTs, *e.g.*, ethylene glycol and urea, which causes the expansion of those layers [18].

3. Halloysite in prophylaxis

3.1. Antiseptics applications

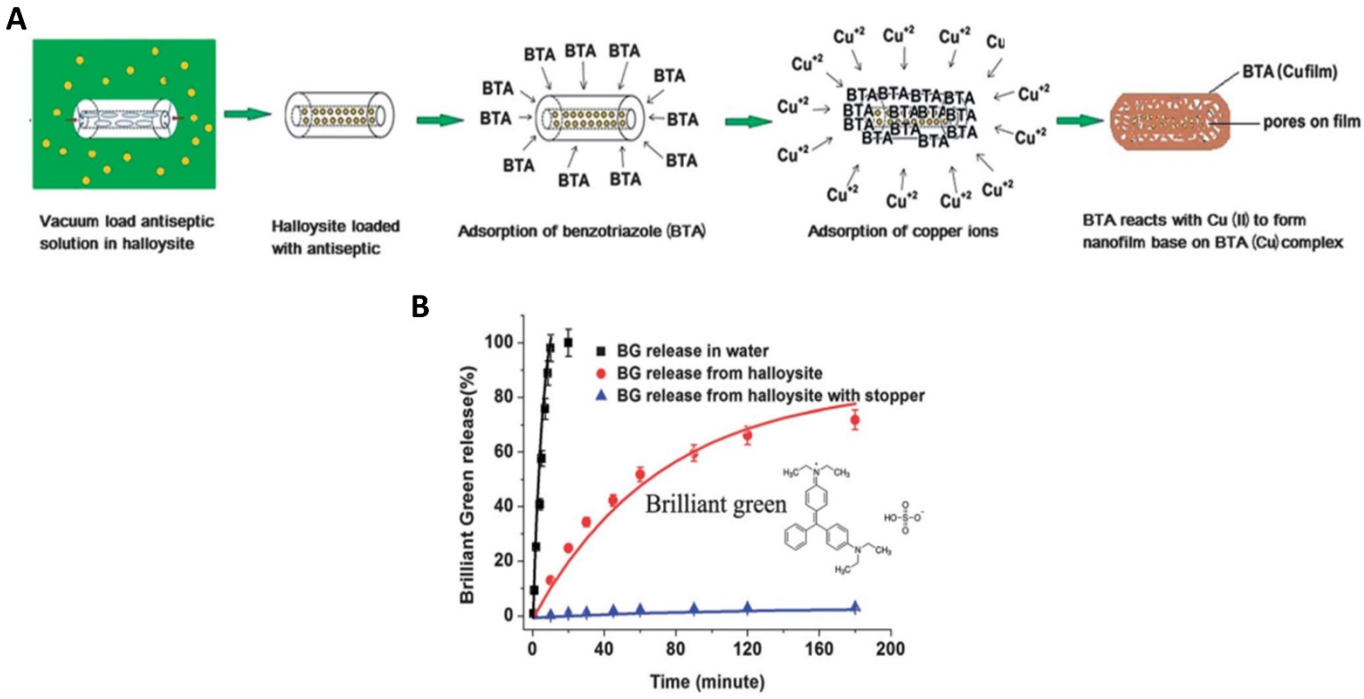

Figure 3. Brilliant green loading inside HNTs with benzotriazole–copper coating (A); and release curves in water of the free drug and encapsulated in HNTs and HNTs with stoppers (B). (A) and (B) reprinted with permission from [6] Copyright 2014, The Royal Society of Chemistry.

Due to the high zeta potential magnitude, HNTs give stable colloids in water, enabling antiseptics encapsulation for antibacterial sprays. Brilliant green, which usually takes 15 min to be fully dissolved, was loaded into the lumen of HNTs at 15 wt. % and it was sustained released over 6 h (**Figure 3**). Benzotriazole–copper complexation at the tubes' ends enabled an extension of the release profile from 50 to 200 h. This was reflected in higher antibacterial efficiency of the loaded brilliant green, extending its action to 72 h when tested against *Staphylococcus aureus* cultures. An extended release of amoxicillin and iodine was also verified after the loading into HNTs [6]. The long-lasting antiseptics release was also demonstrated for chlorhexidine, povidone iodine, and iodine. HNTs were also included in electro spun

poly-e-caprolactone scaffolds allowing for 14 days sustained release, with a superior bacterial growth inhibition [37].

4. Halloysite applications in diagnosis

Clay nanotubes may be used as template for the synthesis and stabilization of different particles, thus enabling the formation of composites with core-shell nanoarchitecture. HNTs-based nanocomposites are being studied as platforms for imaging contrast agents and for the assembling of biosensors.

4.1. Bioimaging

HNTs were used to stabilize photo luminescent labels: lanthanide complexes (*e.g.*, europium(dibenzoylmethane)$_3$ (Eu(DBM)$_3$)) [38,39]; fluorescent carbon dots [33] and cadmium sulfide quantum dots [40,41]. Overall, the clinical application of the biolabels is constrained by their poor photochemical stability, low water dispersibility and toxicity [33,38,39,41]. HNTs-based composites are making labels more stable and less toxic for clinical applications.

Lanthanide complexes (Eu(DBM)$_3$) were attached on the surface of polyethyleneimine (PEI)-modified nanotubes via a ligand exchange (4'-(4-Bromomethylphenyl)-[2,2':6',2'']terpyridine (MPTP-Br)), forming HNTs–PEI–MPTP-Br–Eu(DBM)$_3$. This nanocomposite conferred protection against the photobleaching of the luminescent trivalent lanthanide ions, had lasting luminescence emission, a high quantum yield and showed reduced cytotoxicity on human cervical cancer cells (HeLa cell line) [38].

A multifunctional composite with a PEI-modified HNTs was designed to incorporate luminescent lanthanide complexes (Eu(DBM)$_3$). It proved to have magnetic properties due to the superparamagnetic iron oxide (Fe_3O_4) on the HNTs surface, functioning as a magnetic resonance imaging contrast agent. The *in vivo* magnetic resonance imaging (MRI) analysis of the nanocomposite showed a reduction of the transverse relaxation signal intensity in the normal tissue, enabling the distinction between cancer and normal cells and also demonstrated a reduced toxicity [39].

Carbon dots (CDs), are photoluminescent materials with a diameter of less than 10 nm. HNTs-template was proposed to extended the long-term stability of CDs. The

nanocomposite combined therapy and diagnosis, being recognized as a cancer nanotheranostic agent [33].

Cadmium sulfide (CdS) and cadmium-zinc sulfide (Cd_xZn_yS) quantum dots (QDs) were immobilized onto the clay nanotube surface to produce safer QDs with blue to green colors and surpass photoluminescence intermittency ("blinking behavior"). **Figure 4(A)** illustrates HNT-CdS composites. APTES was used as the grafting agent. This method yielded a high surface coverage, effectively reducing the photoluminescence intermittency. Overall, the immobilization of QDs onto the nanotubes prevents their aggregation, confers resistance to the photobleaching and ensures a good dispersibility in water. HNTs–Azine–$Cd_{0.7}Zn_{0.3}S$ displayed low toxicity in epithelial human prostate cell line (PC-3), constituting a promising cancer intracellular labeling system [41].

4.2. Biosensors

HNTs were applied for enzyme immobilization and glucose detection [42]; capture of tumor cells [43]; detection and quantification of cancer biomarkers [44]; and for the manufacture of ultrasensitive surface plasmon resonance (SPR) sensors [45].

A hybrid composite composed of silver nanoparticles with an average size of 10 nm attached to the surface of silane-modified HNTs were produced through *in situ* chemical reduction of the Ag+ ions. The hybrid nanocomposite represented an alternative platform for the immobilization and electrical wiring of glucose oxidase (GOx), a redox enzyme, that catalyzes the oxidation of glucose into D-gluconolactone with the formation of hydrogen peroxide (H_2O_2). Such immobilization of GOx improved the direct electron transfer forming highly sensitive and stable glucose sensing device for monitoring of diabetes [42].

The controlled evaporation of sodium polystyrene sulfonate (PSS) stabilized HNTs resulted in a stripe-like HNTs pattern, granting the nanotube alignment over a larger area. This rough HNTs surface enabled an efficient tumor cell capture [43].

Following a core-shell architecture, palladium particles were assembled on the surface of polypyrrole (PPy)-coated HNTs producing (HNTs@PPy–Pd). This nanocomposite was designed as the analytical signal label for the quantitative determination of prostate-specific antigen (PSA), a biomarker of prostate cancer.

Figure 4(B) illustrates the PSA sensor fabrication. It showed acceptable selectivity, stability and reproducibility, with a low detection limit of 0.03 pg/mL [44].

The SPR optical technique might be used to construct ultrasensitive label-free sensors [45,46]. HNTs improved significantly the sensitivity of the sensor, taking it to new levels of sensitivity with up to 10^4 nm/refractive index units in a wide refractive indexes range, thereby, constituting devices able to quantify molecules with extremely low molecular weights (~400 Da) [45,46].

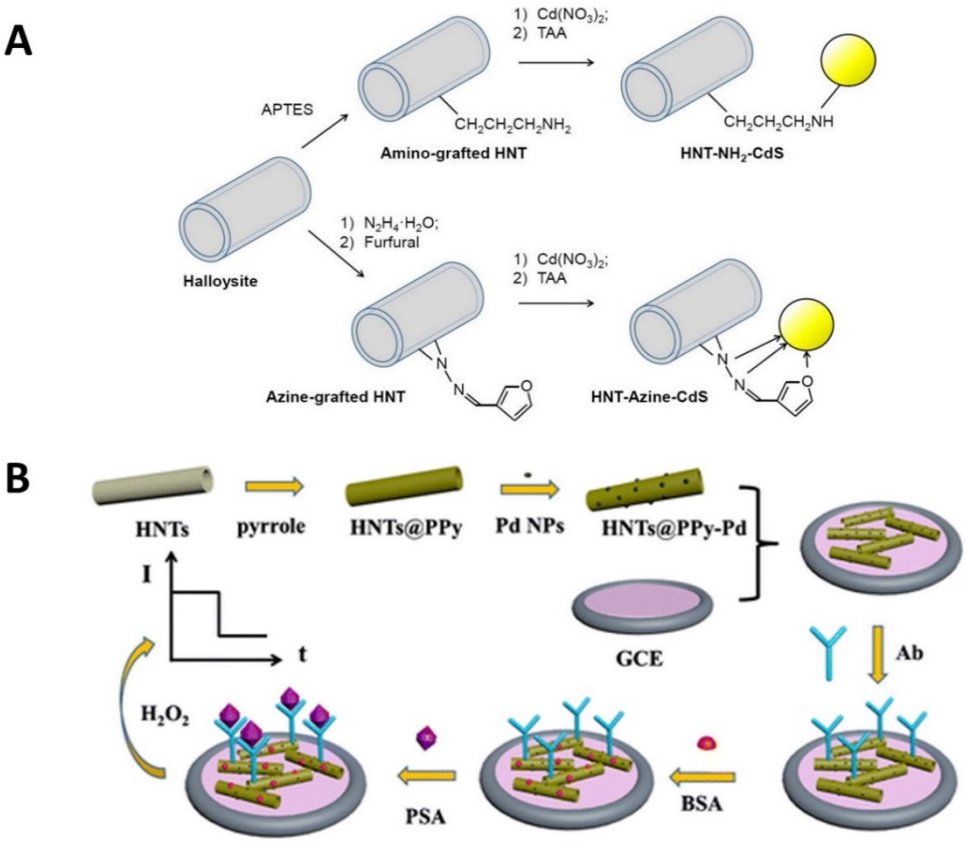

Figure 4. Synthesis of halloysite-CdS composites (A) and the PSA immunosensor fabrication (B). (A) reprinted with permission from [41] Copyright 2018 MDPI; and (B) reprinted with permission from [44] Copyright 2017, Springer Nature.

5. Halloysite applications in therapy

5.1. Drug therapy

5.1.1. Anticancer drugs

HNTs have emerged as a promising system for anti-cancer drug delivery, due to benefits of stable colloidal formation in water and various creams. Their biocompatibility and elongated “torpedo-like” structure endows an easier passage of the cellular membranes without compromising the cell cytoskeleton [7]. Brilliant-green-lumen loaded HNTs coated with dextrin were shown to promote a stimuli-responsive intracellular drug release. Dextrin coating was decomposed by the glycosyl hydrolases present in the intracellular media, promoting targeted delivery. Distinct uptake was found between the culture cell lines tested with higher in adenocarcinoma human alveolar basal epithelial cells (A549) cells in comparison to human hepatoma cells (Hep3b) [7].

HNTs were functionalized with PSS to enhance the biocompatibility and were applied to encapsulate the US Food and Drug Administration-approved photosensitizer indocyanine green (ICG). The functionalized HNTs-PSS-ICG were associated with giant unilamellar vesicles membranes of human breast cancer cells through Pickering effects and accelerated membrane oxidation. Afterwards, the surface modification of HNTs-PSS-ICG with mouse breast cancer MDA-MB-436 cells allowed for a highly efficient *in vitro* membrane disruption capacity and outstanding *in vivo* phototherapeutic effects assessed in a breast cancer mice model [47].

Doxorubicin (DOX) is considered the most powerful anticancer drug, however serious side effects are reported following its *in vivo* administration, requiring new encapsulation delivery systems [48] Gold nanorods were loaded in the lumen, followed by DOX physical adsorption on the surface. The targeting ligand folic acid (FA) was conjugated with bovine serum albumin (BSA), resulting in the Au-HNTs-DOX@BSA-FA nanocomposite (**Figure 5(B)**). The exposition of the functionalized HNTs to near-infrared (NIR) laser irradiation triggered a dual effect by combined photo and chemothermal therapies due to a rise in temperature and drug release. The nanocomposites showed good cytocompatibility and hemocompatibility, with no visible drug toxicity in mice. *In vivo* studies in breast cancer 4T1-bearing mice demonstrated that Au-HNTs-DOX@BSA-FA displayed prominent tumor-targeted efficiency and DOX controlled release pattern, with a prompt photothermal output and the capability to inhibit tumor growth [32].

Soybean phospholipid (LIP) coated nanotubes with DOX were also used to treat gastric cancer. HNTs/DOX/LIP promoted a pH-responsive drug release pattern, with a faster release under acidic environment (pH = 5.4), enabling the targeted drug release. The life-span of tumor-bearing mice was prolonged *in vivo* after the administration of HNTs/DOX/LIP [48].

Folic acid conjugated with chitosan oligosaccharide (COS) assembled with magnetic HNTs were developed as a tumor-targeted camptothecin (CPT) nanosystem (CPT@FA-COS/MHNTs). The pH-dependent drug release pattern proved a triggered release at acidic pH (5.5) rather than neutral and alkaline conditions. A strong cell growth inhibitory effect was encountered against colon cancer cells [49].

Through oral ingestion, HNTs-based tablet formulation was applied through the administration of paclitaxel (PTX). PTX-loaded HNTs enabled a pH-independent controlled drug release pattern, but when further modified by a poly(methacrylic acid-co-methyl methacrylate) (PMMM), the release shifted to basic intestinal conditions. HNTs were identified as a developing compression excipient for tablets formulation [15].

5.1.2. Anti-infectious drugs

Several anti-infectious drugs have been encapsulated into clay nanotubes. A tetracycline HCl, a highly water-soluble antibiotic, was first encapsulated in HNTs. It was mixed with a polymeric carrier (Quitol 651). Tetracycline-loaded HNTs showed a bimodal release pattern, with an initial burst release of 20 %, and then with a slow mode over 30 h [3].

Polymer grafted-magnetic HNTs improved the controlled release of norfloxacin, a cationic antibiotic with adsorption of 72.1 mg/g much higher than native HNTs (30.8 mg/g). Due to the strong interactions, the drug release extended for 60 h [50].

Ciprofloxacin, an antibiotic applied in several bacterial infections, shows a reduced bioavailability as a result of its complexation with physiological iron. In order to overcome this limitation, ciprofloxacin was encapsulated in HNTs functionalized with APTES (70 % drug loading). After 9 h, the ciprofloxacin loaded HNTs exhibited a superior release pattern in phosphate-buffered saline (PBS) buffer. The NH_2 groups of the functionalized HNTs established a strong interaction with the iron, increasing the drug bioavailability [9].

A functionalized HNTs-APTES hybrid was developed for the encapsulation of gentamicin. The APTES was introduced at the surface of HNTs to optimize the gentamicin lumen intercalation. Microbiological activity studies verified high bactericidal activity for *Escherichia coli*, *Staphylococcus aureus* and particularly for *Staphylococcus epidermidis* [24].

Isoniazid is a tuberculostatic drug with low permeability and high solubility that requires higher doses to obtain an efficient therapeutic, resulting in toxicological effects. Isoniazid loaded HNTs were developed. *In vitro* drug release from the nanotubes followed a diffusive kinetic law due to the adsorption-desorption equilibrium between drug in the solution and solid inorganic excipients. Permeability assays exhibited the membrane transport improvements of isoniazid in human colorectal adenocarcinoma cells (Caco-2) cells and the effective cell internalization of the drug. This formulation showed improved stability of isoniazid [14].

A HNTs-based formulation was developed for the controlled release of clotrimazole, an antifungal for the treatment of vaginal and oral candidiasis. Clotrimazole demonstrated an increased interaction with the HNTs lumen in acidic rather than physiological conditions, which was an indicator of a more suitable topical formulation for the vaginal treatment. The HNTs-based hybrid was suggested as a hydrogel filler for topical administration of clotrimazole [51].

5.1.3. Anti-inflammatory and analgesic drugs

APTES-modified HNTs were used for the encapsulation of aspirin (acetylsalicylic acid). The internal lumen modification of HNTs with APTES improved the drug loading [23]. A hydrophobic organic-inorganic HNTs-based system was developed to encapsulate and extend the release time of ibuprofen. The nanotube lumen was enlarged to obtain a higher drug loading capacity; and the surface was hydrophobically modified with TEOS to modify the drug release profile. This formulation extends the drug release profile from 40 h (non-modified HNTs) to 100 h [27]. HNTs have also been functionalized with 3-aminopropyltrimethoxysilane (APTS) and a polyamidoamine dendrimer for the encapsulation of ibuprofen and salicylic acid. This hybrid system was effective in the delivery of low molecular weight drugs that contain carboxyl groups [26].

5.1.4. Corticosteroid drugs

Dexamethasone (DEX) was loaded in the lumen of HNTs in order to control its release [4]. The nanotube surface modification was achieved by LbL deposition of thin layers of chitosan and gelatin. The number of coating layers, the thickness, and the molecular weight were found to impact the release time allowing an extended delivery [29].

5.1.5. Antihistaminic drugs

Diphenhydramine hydrochloride was encapsulated in poly(vinyl alcohol) (PVA)-coated HNTs. The release pattern was found to be pH-controlled, having a smaller release at low pH [52]. Additionally, carboxylated HNTs demonstrated a higher loading capacity and prolonged drug release compared to native HNTs [53]. A nanocomposite containing diclofenac sodium was included into a polycaprolactone/pluronic P123 (PCL/P123) electrospun substrate. The result of the modified-HNTs-containing nanofiber exhibited a superior mechanical performance and thermal stability. Modified composites exhibited a controlled drug release for both drugs. The modified-HNTs-containing microfibers were shown to be a suitable platform for cell growth bone tissue engineering purposes [25].

5.1.6. Cardiovascular drugs

Nifedipine and furosemide were encapsulated in HNTs lumen, achieving a sustained drug release profile for 5-10 h [4]. A stimuli-triggered HNTs-based nanosystem was developed that resulted from the self-polymerization of polydopamine (PDA) in the HNTs surface, followed by their application as fillers in ionically crosslinked alginate gels. The gels were thermally stable and elastic. The HNTs-based gel nanocomposites were applied to encapsulate diltiazem hydrochloride, a calcium channel blocker used for hypertension therapy. *In situ* drug loading demonstrated superior performance [54]. Nifedipine was also encapsulated into HNTs, which were used to obtain composite tablets constituted by 50 wt. % of the drug-loaded HNTs mixed with 45 wt. % of microcrystalline cellulose. Pristine clay nanotubes are excellent tablet excipients, that when composed with nifedipine-loaded HNTs showed a close to zero order drug release pattern up lasting up to 20 h. The system

showed an enhanced photostability, suggesting the use of HNTs for oral formulations [55].

5.1.7. Biopharmaceutical agents

The use of HNTs for intracellular delivery of biopharmaceutical agents, such as nucleic acids, proteins (enzymes and insulin) have taken place. The adsorption of biomacromolecules in the nanotubes confers protection against degradation, enabling a prolonged therapeutic activity and a reduction of doses, towards essentially for anticancer effects [1,7,56].

The biomacromolecules net charge and the nanotube's inner and outer surface charges dictate the way they are absorbed. In the case of proteins, these electrostatic interactions are mainly influenced by the pH of the protein solution [1,56]. The negatively charged HNTs surface area is larger, thus the adsorption of positively charged proteins is a straightforward process. Negatively charged globular proteins with *ca.* 2-8 nm diameter were loaded into the positively charged inner lumens (diameter 15-20 nm). Pepsin, urease, peroxidase, lipase, laccase, and GOx are examples of negatively charged enzymes that have been loaded into the lumen of HNTs, **Figure 5(A3)**. The adsorption for these enzymes was 6-7 wt. %, a quite low value in comparison to the adsorption of positively charged proteins onto the HNTs outer surface, which displayed 15–25 wt. %. The release profile of negatively charged enzymes loaded in HNTs showed a faster initial burst followed by a prolonged 10 h release pattern. An advantage of the loading of negatively charged proteins is that more than half of the enzymes permanently remains in the inner cavity of HNTs, creating functional confined enzymatic nanoreactors.

Laccase, an oxidase with anti-proliferative properties, was immobilized via electrostatic interactions in HNTs [56]. In a recent study, HNT immobilization of laccase occurred through a covalent interaction promoted by a crosslinking agent, glutaraldehyde [57]. This nanocomposite constitutes a promising theranostic platform to be applied in cancers overexpressing estrogen receptors, such as cervical or breast cancers [57]. Ribonucleases might also be applied in enzymatic cancer therapy due to their anticancer potential [58]. Binase, a cationic ribonuclease at pH 6-8 (pI = 9.5), was immobilized onto the HNTs in order to stabilize the enzyme and prolong its release. It was suggested that binase, which shows a cationic and a

hydrophobic nature at physiological pH, could be adsorbed on both the outer surface and inner lumen of HNTs. The complex of binase–HNTs showed *in vitro* anticancer effect towards human colon adenocarcinoma cells (60% reduction compared to 25% reduction of cells viability promoted by the exposure to free binase), inhibiting the *RAS* oncogene downstream signaling pathway [58].

Insulin is a peptide hormone that displays a negatively net charge at neutral and alkaline pH, having electrostatic affinity to the HNTs hollow lumen for sufficient loading. The complexes with 2.6 wt. % of insulin loading showed a 20 % release in the first 10 h followed by a prolonged release during 120 h. HNT-nasal spray may be an alternative for sustain insulin delivery [59]. A different possibility for the oral administration of insulin is the use of NiMOS, which consists of the protein loaded HNTs entrapped in microgels produced by the prilling method (**Figure 2(D)**) [13].

Therapeutic nucleic acids hold phosphate groups responsible for their anionic nature at physiological pH. Affinity to the aluminol (Al–OH) groups of HNTs lumen may stabilize and protect nucleic acids from degradation by DNases and RNases, enhancing their stability and intracellular uptake [1,22]. The use of a ball milling led to the adsorption of anionic DNA onto HNTs surface with zeta potential increase from −30 to −65 mV. These results are due to the neutralization of the internal positive charges with insertion of DNA inside HNT, and, probably partial coating of the tube's outside. The DNA-modified HNTs displayed an improved water dispersibility and longtime colloidal stability [60]. The two strategies used for loading and intracellular delivery of therapeutic nucleic acids that inhibit gene expression, antisense oligodeoxynucleotides (ASODNs) and small interfering RNA (siRNA), were based on: (i) the hydrophobization of the HNTs surface with APTES, or (ii) its functionalization with cationic polymers, such as PEI [22,61,62].

LbL-method was used for PEI-coating forming PEI-HNTs-siRNA-QDs complexes. These complexes showed a high transfection efficiency in human pancreatic cancer cells (PANC-1 cell line) (95.6 %) and stabilized QDs enabling the visualization of the process. The HNTs-based nanocomplexes effectively silenced the gene of interest (*survivin*) inhibiting the survival of cancer cells, displaying their *in vitro* potential application as cancer gene therapy vectors [61]. In order to potentiate green fluorescence protein labeled DNA deliver without causing cell injury and inflammation, shortened HNTs with 200 nm length were used. In human cervical

epithelial adenocarcinoma cells (293T and HeLa cell lines) short PEI-grafted HNTs (PEI–g–HNTs) exhibited higher DNA transfection efficiency and reduced toxicity as compared with complexes formed by PEI/DNA coacervates [62].

Recently, the first *in vivo* evidence of HNTs used as nanovectors for gene delivery was given in Long, Wu [63]. Polyamidoamine (PAMAM) dendrimers were grafted onto the surface of HNTs conferring a positive surface charge (+19.8 mV), that enabled the complexation with the vascular endothelial growth factor (VEGF) siRNA (siVEGF). The PAMAM–g–HNTs–siVEGF showed a cellular uptake efficiency of 94.3 % towards human breast cancer MCF-7 cells and reduced by 78.0 % the expression of VEGF messenger RNA (mRNA) inducing the apoptosis of cancer cells. The intratumoral injection of the PAMAM–g–HNTs–siVEGF suspension (0.7 mg/kg dose) in breast cancer 4T1-bearing BALB/c mice inhibited tumor volume and angiogenesis promoting tumor necrosis [63].

5.1.8. Natural compounds

Resveratrol was loaded into HNTs showing a 48 h release curve, which was further delayed by LbL polycation/polyanion coating. *In vitro* studies on breast cells (MCF-7) proved that the LbL functionalization decreased the clay nanotube self-toxicity, and this formulation increased apoptosis [8].

Curcumin, another actively researched polyphenol, was encapsulated in HNTs whilst the surface being functionalized with poly(N-isopropylacrylamide) (PNIPAAM), a temperature-responsive material. The colloidal stability and the wettability were triggered by temperature change. Moreover, under gastrointestinal simulated conditions, a targeted enteric drug release was found, preventing the degradation of curcumin the gastric medium [64]. A dual-stimuli responsive prodrug of curcumin was developed, through the linkage of cysteamine to the outer surface of HNTs, creating a reduction-responsive system. The presence of intracellular glutathione or an acidic environment triggered the curcumin release. A high cytotoxicity was found in two hepatocellular carcinoma cell lines, where the concentration of glutathione is high, as compared to the free drug. This approach is promising towards hepatocellular carcinoma therapy [34].

Curcumin was also encapsulated in HNTs by the synthesis of a NIR-light and hybrid nanosystems. First, curcumin-gold nanoparticles were deposited. The presence of

gold conferred NIR-light responsive. The curcumin-gold nanoparticles-HNTs were coated by chitosan, which attributed pH-responsive features. Curcumin's release pattern was pH-dependent, triggered in acid media (pH = 5.5) rather than extracellular conditions (pH = 7.4). The formulation anticancer activity was found on MCF-7 cells [65].

The HNTs surface modification with poly(methyl vinyl ether-co-maleic acid) (PMVEMA) promoted interactions with the intestinal Caco-2/HT29-MTX cells/epithelia, increasing the curcumin permeability by 13 times. This was considered as a viable approach for the delivery of curcumin and low soluble drugs by the oral route [66].

Multifunctional quercetin loaded HNTs with targeting and imaging capabilities were also developed. This was achieved by linking covalently 6-arm polyethylene glycol-amine branches (g-PEG) to the surface of HNTs. Photoluminescent features were then introduced by the conjugation of biocompatible carbon dots. Simultaneously, biotin was conjugated to g-PEG in order to achieve tumor tissue targeting and higher cellular uptake. Quercetin was then adsorbed to the system. A sustained and pH-responsive drug release was found. The nanocomposite displayed low toxicity and high biocompatibility, together with an enhanced anticancer activity against HeLa cells [33].

HNTs may be also applied for the encapsulation of volatile substances since the loading process does not use high temperatures. Carvacrol, an essential oil endowed with antimicrobial properties was loaded into hydrophobized HNTs to enhance its thermal stability, which enables low-density polyethylene melted compounding. Remarkable antimicrobial properties were reported against *E. coli*, *Listeria innocua* and *Alternaria alternate* [67]. Thyme essential oil was also encapsulated into the lumen of HNTs followed by end-capping or LbL external coating. A release pattern was identified over many days, and high antibacterial effect was reported against *E. coli* [30].

5.2. Tissue engineering and regenerative medicine

In the field of tissue engineering and regenerative medicine, HNTs were incorporated in 3D scaffolds to promote cells adherence and proliferation in complex hierarchical structures (*e.g.* trophoblast vesicles).

HNTs were incorporated in a porous hydrogel scaffold consisting of three biopolymers: chitosan, gelatin, and agarose [35]. The addition of HNTs resulted in an upgrade of the mechanical, thermal and wetting properties of the scaffold. The *in vitro* tests performed with several human cancer cell lines have suggested that the scaffolds stimulated cell adhesion and proliferation simultaneously whilst maintaining cell viability. The scaffolds fostered the neovascularization process, completely restoring the blood flow in the rat's connective tissue [68]. Recently, a polylactic acid (PLA) stripped pattern was produced and then covered with a layer of PDA to enable the nanotube attachment for cell orientation, which is important in cell culture scaffolds and biosensors [69]. Such HNTs-coated PLA pattern was suitable for the adhesion and proliferation of human mesenchymal stem cells.

The delivery of 4-aminopyridine (4AP)-loaded HNTs promotes nerve impulse conduction and enhances the nerve regeneration after peripheral nerve injury [70]. This porous drug delivery composite was formed by a chitosan scaffold consolidated with 4AP-loaded HNTs, showing an aligned micro-channel porosity. *In vitro* experiments used the potassium-channel blocker loaded in the HNTs enhanced the nerve regeneration through the expression of nerve growth factor, myelin protein and brain derived neurotrophic factor. The surgical viability and biocompatibility of this system were assured in a rat model that had defects in the sciatic nerve.

5.2.1. Wound healing

HNTs that are loaded with antibiotics and embedded in distinct types of biopolymer matrices have the potential for wound dressings with anti-inflammatory and antimicrobial activities. Ciprofloxacin dispersed in a gelatin-based matrix and polymyxin B sulfate-loaded HNTs arranged uniformly into the matrix were designed for wound-healing [71]. The combination of two antibiotics in the nanocomposite showed a synergetic antimicrobial effect [36]. Drug delivery was accomplished using silane modified HNTs loaded with metronidazole and doped in polycaprolactone/gelatin electrospun microfibers, forming guided tissue regeneration/guided bone regeneration (GTR/GBR) membranes that contained *ca.* 25 wt. % of metronidazole. The halloysite-doped membranes were biocompatible, inhibited fibroblast ingrowth, as well as the colonization of anaerobic *Fusobacterium nucleatum* due to the prolonged release of metronidazole for 20 days.

HNTs were also incorporated in a pour powder formulation for wound-healing [72]. First a nanocomposite based on HNTs and chitosan oligosaccharide was developed. The powder was applied for 7 days in burns induced on the rat's dorsum and improved the healing process.

5.2.2. Bone repair

For bone repair purposes, 5-8 wt. % of gentamicin was loaded in HNTs and uniformly deployed into a polymethyl methacrylate (PMMA) bone cement [73]. The PMMA cement reinforced with 5-7 wt. % of the nanotubes exhibited optimal mechanical and adhesive properties. HNTs–PMMA cement prolonged the release of gentamicin during 300-400 h without interfering with the bone mechanics. The formulation displayed excellent adhesive properties, demonstrating potential for orthopedic replacement surgeries.

HNTs might be applied in the immobilization of enzymes responsible for the biomineralization process forming inorganic substrates that yield bone minerals [74]. One of those enzymes is alkaline phosphatase (ALP) which has been loaded in HNTs. The immobilization of ALP in HNTs and the use of calcium glycerophosphate promoted the biomineralization process forming hydroxyapatite.

Scaffolds used in the differentiation of stem cells to osteoblasts show applications in bone tissue engineering. HNTs surface functionalized with supermagnetic iron oxide, chitosan and two-dimensional calcium phosphate nanoflakes formed a scaffold improving the osteogenic potential of human adipose tissue-derived mesenchymal stem cells (hADMSCs) [75]. This multifunctional scaffold enabled the adherence, proliferation, and osteogenic differentiation of hADMSCs, enhancing osteoconduction.

HNTs with specific oriented patterns on solid substrates using the shearing brush method and thermoevaporation were developed [76]. HNTs were aligned in a strip-like pattern with a periodicity of 30–100 μm with the thickness being proportional to the tubes' concentration. This oriented structure was employed in the culture of human bone mesenchymal stem cells.

5.2.3. Dental repair

Triclosan-loaded HNTs were applied in esthetic resin composites (based on methacrylate) allowing an efficient dentin remineralization and a sustained release of

the antibiotic [77]. The restorative treatments need to be time extended. Diminishing the durability of the dental restoration might lead to the development of secondary caries [78]. The incorporation of doxycycline-loaded HNTs in a dental restorative adhesive enhanced the durability of the resin/dentin adhesive system, through the suppression of the matrix metalloproteinases (MMPs) activity, constituting a viable clinical strategy to optimize minimally invasive dental treatments.

HNTs were also doped in endodontic resins to disinfect and seal the root canal in order to avoid bacterial recolonization, improving the efficacy and longevity of the endodontic treatments [79]. Alkyl trimethyl ammonium bromide (ATAB), a quaternary ammonium compound, was loaded in the HNTs and incorporated in a resin showing a high antibacterial activity against *Enterococcus Faecalis,* the main bacteria involved in endodontic infections.

5.2.4. Hair surface engineering

Hair surface engineering with clay nanomaterials has shown potential in three areas: human medicine, veterinary medicine, and cosmetics [10]. In human and animal hair cuticles, an aqueous nanotube suspension formed a homogeneous multilayer coverage with *ca.* 3 μm thick that endured for 10 shampoo washings, **Figure 5(C, D).** The hollow lumen of HNTs enabled loading of a hair dye (2-hydroxy-1,4-naphthoquinone) and effectively colored both pigmented and grey hair, representing a less toxic and environmental-friendly alternative to the conventional hair coloring. Regarding therapeutics, two hydrophobic drugs were loaded into the tube's hydrophobized lumen. Permethrin, which is used to treat scabies and lice, and minoxidil, to treat alopecia. The biocide effect of permethrin was confirmed *in vivo*, in *Caenorhabditis elegans* nematodes. HNTs-based hair coating strategy might be favorable for the treatment of alopecia and parasitic infestations, such as pediculosis and scabies.

6. Toxicity and nanosafety

The toxicological profile of this clay nanomaterial has been investigated mostly in *in vitro* and few works - in *in vivo* conditions. A high level of biocompatibility and low cellular toxicity of HNTs have been reported in cervical adenocarcinoma (HeLa) and breast cancer cells (MCF-7). HNTs are non-toxic for concentrations as high as 100

μg/mL [80]. In human umbilical vein endothelial cells (HUVECs) and MCF-7 cells, HNTs at ≤ 200 μg/mL displayed biocompatibility, low toxicity, and the capability to be absorbed by cells [81].

Polycations have been extensively employed for HNTs surface modifications. *In vitro* testing cytocompatibility, uptake, and toxicity showed alterations in cellular nuclei morphology [82]. The blood compatibility of HNTs was also investigated for higher concentrations of HNTs (0.5 mg/mL). Red blood cells (RBCs) changes and aggregation were detected in PBS. HNTs can stimulate complement activation, affect coagulation pathways, and alter the thromboelastogram in a non-severe way. When tested in the precursor macrophage cell line (RAW 264.7), HNTs follow a concentration-dependent cellular uptake [83].

HNTs are potential inducers of sub-chronic toxicity in mice after inhalation. A blockade of autophagic processes resulting in p62 accumulation was detected, which conducted to apoptosis, oxidative stress, and inflammatory responses. Results were reversed when trehalose was orally administered to mice, by reducing the levels of p62, favoring autophagy and leading to lower HNTs-related toxicity [84]. Additionally, HNTs- liver toxicity was studied in mice at body weights of 5, 50, and 300 mg/kg. The results showed that only the mice treated with higher HNTs concentrations (300 mg/kg) displayed growth inhibition and liver toxicity results, but smaller concentrations were safe. The findings were justified by aluminium accumulation and consequent oxidative damage [85]. Mice were administered intragastrically with two different concentrations of HNTs at 5 or 50 mg/kg throughout 30 days, in order to access possibilities of pulmonary toxicity. HNTs accumulation and high aluminium levels in the lungs were found in the mice treated with high nanotube doses [16]

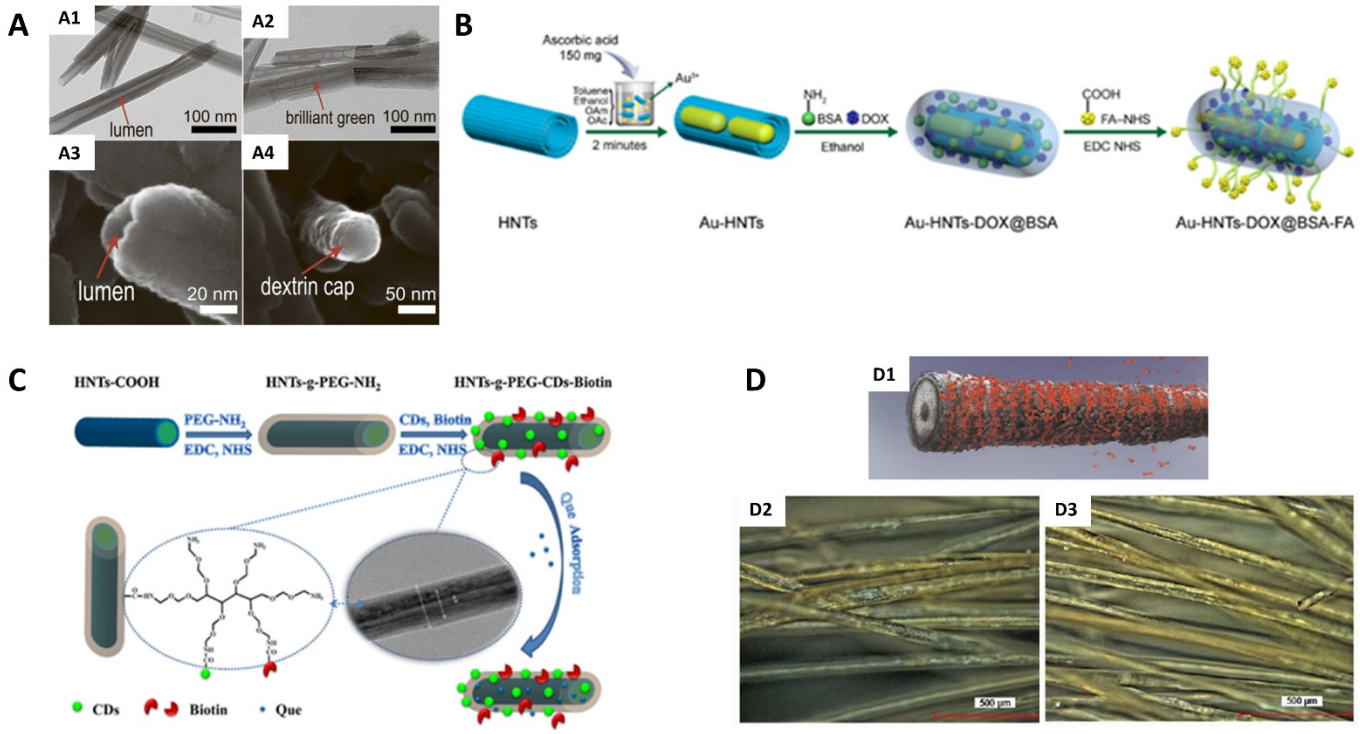

Figure 5. TEM image of pristine HNTs (A1); TEM image of brilliant green-loaded HNTs (A2); SEM image of an end of pristine nanotube with open lumen (A3); and SEM image of a dextrin cap on the end of the functionalized nanotube (A4). Synthesis processes of Au-HNTs-DOX@BSA-FA (B). Illustrations of chemical structure and preparation procedure of HNTs-g-PEG-CDs-Biotin followed by Que adsorption (C). Self-assembly of halloysite clay nanotubes on the hair cuticle (D1) and high definition images of hair coated with orange lawsone loaded halloysite (D2-D3). (A) reprinted with permission from [7] Copyright 2015, Nature Publishing Group. (B) reprinted with permission from [32] Copyright 2019, American Chemical Society. (C) Reprinted with permission from [33] Copyright 2018, Elsevier. (D) reprinted with permission from [10] Copyright 2018, Royal Society of Chemistry.

No significant toxic effects were detected on *C. Elegans* microworms, as attributed to the minimal uptake by intestinal epithelial cells, with negligible transportation to other tissues [86]. In *Paramecium caudatum,* the clay nanoparticles studied (montmorillonite, halloysite, kaolin, and bentonite) induced little or no toxicity, whereas significant toxicity was found for the graphene oxide nanomaterials. In fact, concentrations up to 10 mg/mL of HNTs were considered safe showing no harm for

the microhabitat of this protozoan [87]. Further nanotoxicity studies were conducted in zebrafish exhibited no acute toxicity and suggested HNTs as a safe nanomaterial for concentrations as high up to 25 mg/mL. No cardiotoxicity was observed [81]. HNTs are considered relatively safe and biocompatible nanomaterials, bearing reduced potential for toxicity.

7. Conclusions

Halloysite is a natural, inexpensive and ready-to-use nanomaterial. Clinically, it promises the development of new delivery systems for all stages, such as prophylaxis, diagnosis, and therapy. HNTs are constituted by rolled aluminosilicate sheets that create a structure with a positively charged hollow lumen and a negatively charged surface allowing for good dispersibility in water, alcohol and polar polymers. The empty lumen with 15-20 nm diameter represents a technological opportunity for drug encapsulation. This structure enables drugs to be loaded into the lumen by 10-20 wt. %, extending the drug release pattern up to 10-20 h. Acid etching enhances the lumen volume capacity to *ca.* 30 %. A selective drug immobilization is possible regarding the opposite inner/outer net charge of the tubes' surface.

The functional versatility of HNTs include silane modifications, LbL self-assembly, TEOS and OTES. These includes targeting moieties, such as folic acid or biotin and enzymatically responsive materials (dextrin end-stoppers); or establishing pH-responsive bonds at the tubes' surface (cysteamine disulfide bonds). This enables a selective cellular internalization and triggered drug release for anticancer applications. The rod-shape is another distinguished feature of HNTs that improves the cellular internalization.

Halloysite was already used for prophylaxis, diagnosis and therapy. A variety of active pharmaceutical ingredients have been encapsulated in the clay nanotubes. The pharmaceutical applications available include the encapsulation of anticancer, anti-infectious, anti-inflammatory, analgesic, corticosteroid, antihistaminic, and cardiovascular drugs. Additionally, biopharmaceutical agents and natural compounds for therapeutics were formulated with this nanoclay.

HNTs are excellent platforms for enzyme and DNA immobilization in clinical diagnostics and therapy. The nanoclay encapsulation preserves the bioactivity of enzymes, give them protection against protease degradation and enable recovery of the enzymatic degradation byproducts. HNTs improved the application of enzymes in cervical, breast and colon cancer. HNTs have emerged as less toxic non-viral nucleic acids vectors when compared to carbon nanotubes or cationic polymer-nucleic acids complexes. The high transfection efficiency and gene silencing foresee HNTs application as vectors for gene therapy.

HNTs have been successfully applied in tissue engineering and regenerative medicine, specifically in wound healing, bone and dental repair, as well as in hair surface engineering. The creation of biomimetic architectures fostered adhesion, proliferation, and differentiation of stem cells. HNTs enhance scaffolds mechanical properties, as well as the water uptake.

Loading bioactive compounds into the nanotube lumen enables the design of bioactive matrices, membranes, or pour-powders that release antibacterial drugs promoting faster wound-healing precluding infections. Similarly, the inclusion of prophylactic antibiotic-loaded HNTs in bone cement demonstrated clinical application for orthopedic replacement surgeries. The use of HNTs for the immobilization of enzymes associated with the bone biomineralization process is a major breakthrough in bone repair. In dentistry, incorporation of HNTs in resins exhibits applications in restorative dental caries and endodontics treatments. Hair coating with HNTs was used for the treatment of human and veterinary hair pathologies, such as parasitic infestations. Applying HNTs into polymeric matrices, gels, creams, sprays, dental resins, bone implants, hair treatments by self-assembly, tissue scaffolds, and electrospun nano/microfibers, enables the development of several pharmaceutical dosage forms. HNTs are also a “ready-to-use” excipient material for oral tablets, as well as a filler for gels.

Human exposure to this nanoclay materials showed low toxicity and elevated biocompatibility, as demonstrated by several *in vitro* and *in vivo* models (with different cells lines, microworms, infusoria, fishes, mice and rats). Among many clays tested, HNTs displayed the lowest cytostatic activity, and are much safer than carbon nanomaterials, but one has to be caution using high concentrations of the nanotubes (above 0.5 mg/mL). Being considered as “green”, biofriendly and safe

natural biomaterial, halloysite clay nanotubes require definite recommendations regarding human pharmacy toxicity limits.

8. Expert opinion

Relevant clinical applications of HNTs have been presented, particularly, the nanoscale-sized empty lumen allowing for 10-20 wt. % drug and protein loading, tubes' high length to diameter ratio, different inner/outer surface charge, functionalization methods, good dispersibility in water and polymers, biocompatibility, low cost and natural availability. The drugs encapsulated in HNTs have a sustained 10-20 h release, which may be extended to 100 h with the tube's end capping or the inclusion of the nanotubes into a polymeric matrix. Drug-loaded HNTs may be used for tablet formation and for different topical formulations, including creams, powders and spray. These features present advantages over other drug-clay composites, including kaolin, sepiolite and montmorillonite.

Based on animal models, halloysite is the safest clay material, and is much less harmful than carbon nanomaterials, including carbon nanotubes. Halloysite is a safe clay allowing for "green" processing, which eliminates concerns on complex and hazardous productions methods, as well as associated production costs. The tubes' surface silanization offers an avenue for the development of functional composites. That characteristic enables the ability to attain several types of release profiles, varying from a simple sustained release to a stimuli-triggered release profile, including pH-, enzymatically-, NIR-, and/or redox-responsive release patterns. Therapy combinations such as photodynamic, photothermal, chemotherapy, and gene therapy are possible with HNTs formulations.

HNTs are promising for prophylaxis in high-risk work environments, especially to fight the worrying nosocomial infections. HNTs usefulness is displayed in tissue engineering and bandages for wound healing effects. HNTs encapsulation surpasses the physicochemical limitations of biopharmaceuticals limited due to their high molecular weight and short half-life. We estimate that novel enzymes and RNA interference macromolecules will be stabilized with these nanotubes. DNA/RNA-HNTs therapeutic vectors constitute a very promising research area.

The application of HNTs for tissue engineering and regenerative medicine has been the focus of recent research. These nanotubes reinforced scaffolds and membranes producing multifunctional sustained drug delivery systems. The biomimetics strategy has led to scaffolds with targeted architectures and the same cells might be applied in different clinical contexts due to a direct link between structure and function, like in HNTs oriented patterns.

Diverse clinical applications are expected with HNTs incorporation in dose-dependent medications. The most viable formulations will be topical formulations, specifically, creams, gels, sprays, bone and dental cements, not only for human but also veterinary use. The focus of topical application arises on the fact that this nanomaterial is not easily biodegradable, limiting its use in intravenous formulations, due to the risk of aluminium accumulation in the presence of high concentrations, but not disqualifying HNTs for intramuscular administration and short-duration treatments. HNTs are considered by the Environmental Protection Agency (EPA) as 4A listed material, thereby seen as natural, nontoxic, and biocompatible. A weakness is the lack of comprehensive studies to assess the efficacy, specificity, pharmacokinetics, toxicity, and safety of HNTs, which should be addressed in humans rather than *in vitro* and in animals *in vivo* models.

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Declaration of interest

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List of abbreviations

4AP	4-aminopyridine
AFM	Atomic force microscopy
ALP	Alkaline phosphatase
APTES	3-aminopropyltriethoxysilane
APTS	3-aminopropyltrimethoxysilane
ASODN	Antisense oligodeoxynucleotide
ATAB	Alkyl trimethyl ammonium bromide
BSA	Bovine serum albumin
CD	Carbon dot
COS	Chitosan oligosaccharide
CPT	Camptothecin
DEX	Dexamethasone
DOX	Doxorubicin
EPA	Environmental protection agency
$Eu(DBM)_3$	$Europium(dibenzoylmethane)_3$
FA	Folic acid
GOx	Glucose oxidase
g-PEG	Poly (ethylene glycol)-amine branches
GTR/GBR	Guided tissue regeneration/guided bone regeneration
HNT	Halloysite clay nanotube
ICG	Indocyanine green
LbL	Layer-by-Layer

LIP	Soybean phospholipid
MMP	Matrix metalloproteinase
MPTP-Br	4’-(4-Bromomethylphenyl)-[2,2’:6’,2’’]terpyridine
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NiMOS	Nanoparticles-in-microgel oral systems
NIR	Near-infrared
OTES	Octyltriethoxysilane
P123	Pluronic P123
PAMAM	Poly (amidoamine)
PBS	Phosphate-buffered saline
PCL	Poly (caprolactone)
PDA	Poly (dopamine)
PEI	Polyethyleneimine
pI	Isoelectric point
PLA	Poly (lactic acid)
PMMA	Poly (methyl methacrylate)
PMMM	Poly (methacrylic acid-co-methyl methacrylate)
PMVEMA	Poly (methyl vinyl ether□co□maleic acid)
PNIPAAM	Poly (*N*-isopropylacrylamide)
PPy	Poly (pyrrole)
PSA	Prostate-specific antigen
PSS	Poly (styrene sulfonate)
PTX	Paclitaxel
PVA	Poly (vinyl alcohol)

QD	Quantum dot
RBC	Red blood cell
SEM	Scanning electron microscopy
siRNA	Small interfering RNA
siVEGF	Vascular endothelial growth factor small interfering RNA
SPR	Surface plasmon resonance
TEM	Transmission electron microscopy
TEOS	Tetraethoxysilane
VEGF	Vascular endothelial growth factor
β-CD-(SH)$_7$	Per-thiol-β-cyclodextrin