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HALLOYSITE-BASED NANOSYSTEMS FOR BIOMEDICAL APPLICATIONS

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Abstract—Halloysite nanotubes (HNTs) are hollow clay nanotubes in the nanometer size range, made up of double-layered aluminum silicate mineral layers. HNTs represent an extremely versatile, safe, and biocompatible nanomaterial, used in a wide range of applications in biomedicine and nanomedicine. For example, they are used as transporters for the controlled release of drugs or genes, in tissue engineering, in the isolation of stem cells and cancer cells, and in bioimaging. Consequently, the assessment of the biocompatibility of HNTs has acquired considerable importance. In recent years, HNT composites have attracted attention due to their improved biocompatibility, compared to HNTs, suggesting potential for applications in tissue engineering or as vehicles for drugs or genes. In this review, recent advances in the application of HNTs and HNT composites in biomedicine are discussed to provide a valuable guide to scientists in the design and development of viable, functional bio-devices for biomedical applications.

Keywords—Biomedicine · Drug delivery · Gene Delivery · Halloysite Nanotube Composites · Nanotubes · Tissue Engineering

INTRODUCTION

Significant progress has been made with nanotechnologies which are applied in many fields of the food and pharmaceutical industries and in environmental technology, science, and energy, etc. (Bayda et al., 2020). Interest in tubular nanoformulations has grown in recent years, due to their potential application in various fields of nanomedicine (Patra et al., 2018; Liu et al., 2019). Many nanomaterials are available naturally or are produced artificially. Among these, halloysite nanotubes (HNTs) are abundantly available in nature (economic and sustainable deposits) or can be produced synthetically (Jin et al., 2015). In nature, HNTs are present in soils and rocks exposed to atmospheric agents typical of humid tropical and subtropical areas, and particularly in volcanic ash and tephra in a variety of climates (Massaro et al., 2017). Natural deposits of HNTs are found in Japan, New Zealand, China, the United States, Korea, Japan, Turkey, Brazil, and France. Halloysite clay is available, therefore, in large quantities (many thousands of tons) from various mines, at low cost, making halloysite an extremely competitive alternative to carbon-based nanomaterials (Jawaid et al., 2016). The physical properties of the HNTs, such as length, tube-wall thickness, internal radius, dispersion, and zeta potential, are influenced by the charatcteristics of the deposit, however (Yuan et al., 2015). Inhomogeneous HNTs with lengths between 100 nm and 2 mm are available commercially. Long nanotubes have been shown to act as inducers of inflammation and cell damage (Wang et al., 2018). The size of the nanoparticles (NPs) has an effect on how the body's cells 'see' them and, therefore, determine their distribution, toxicity, and ability to reach the desired site. For example, in order to overcome the blood-brain barrier (BBB) for the administration of therapeutic agents to the Central Nervous System, NPs between 2 and 200 nm in size accumulate more efficiently in the brain according to Persano et al. (2021). In addition, the size of the NPs influences their biological fate, i.e. NPs with a size of >200 nm activate the lymphatic system, with consequent rapid removal from circulation (Maisel et al., 2017). Consequently, NPs with a diameter of <200 nm are more suitable as nano-platforms for the administration of therapeutic agents; such particles undergo endocytosis to a much greater extent than larger NPs (Behzadi et al., 2017). Heterogeneity in terms of the size of HNTs does not hinder their application, because smaller-diameter nanotubes can be produced by ultrasonic treatment of larger HNTs (Fig. 1) (Rong et al., 2016).

Halloysite particles occur in various forms, from short tubular clay structures to spheroidal structures and even flat and other nanostructures, but the most common are elongated tubular structures (Hillier et al., 2016). HNTs are produced by winding on themselves of flat kaolinite layers (aluminosilicate, empirical formula $Al_2Si_2O_5$ $(OH)_4{\cdot}nH_2O$), 15–20 layers thick, giving rise to a hollow, porous tubular structure, with a large length/diameter ratio, a lumen diameter of between 10 and 40 nm, and an external (total) diameter between 40 and 70 nm (Daou et al., 2020). HNTs typically range between 0.2 and 2 μm long (Fig. 2a). Some authors claim that HNTs have a two-layered structure (Chen et al., 2018). In its pure form, halloysite is white in color, but sometimes the presence as impurities of traces of transition metal ions, which replace the Al and Si atoms, give rise to different colors ranging from yellow to brown and sometimes green (Saif et al., 2018). Almost pure HNTs have been identified in Utah (USA) while most natural HNTs have some impurities, such as quartz, kaolin, chlorite, illite, gibbsite, feldspar, salts, and metal oxides (e.g. iron oxide, copper oxides, oxides of titanium, and calcium oxides). These impurities can

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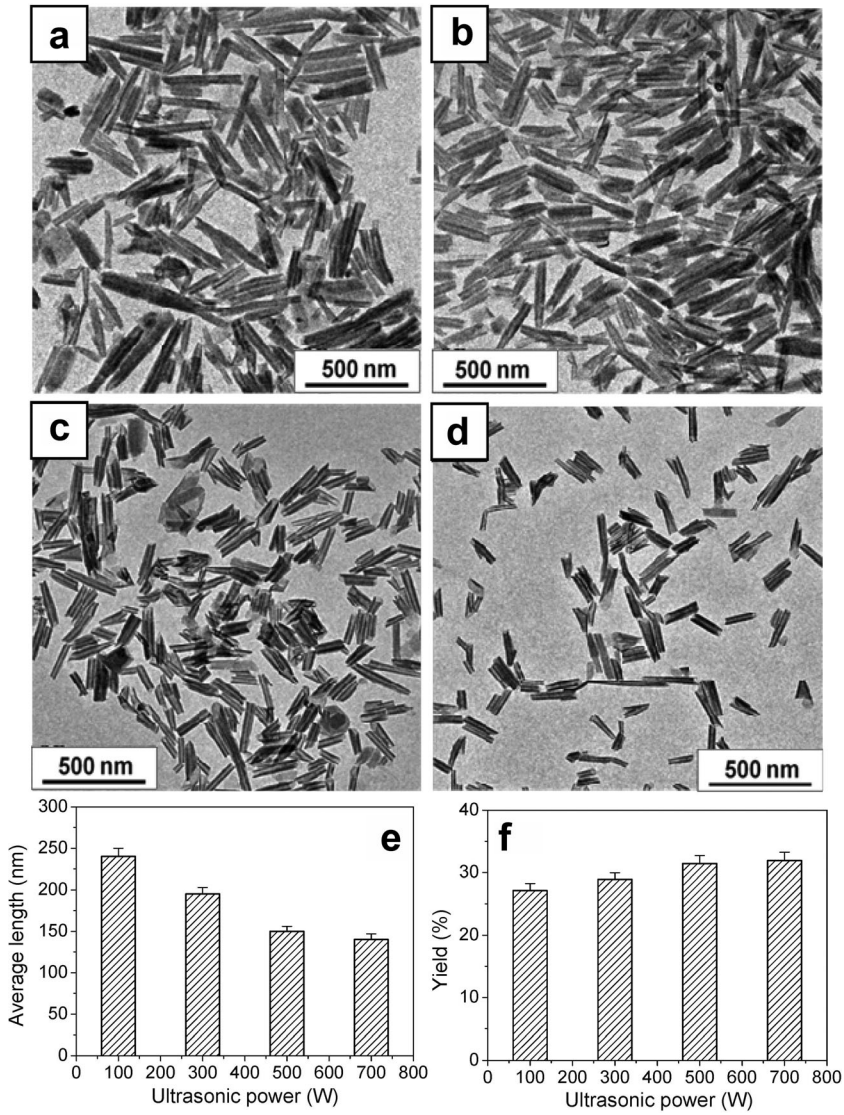


Fig. 1. TEM images of HNTs obtained by ultrasound for 600 s at **a** 100 W, **b** 300 W, **c** 500 W, and **d** 700 W, respectively. **e** Effect of ultrasonic power on the average length of HNTs. **f** Effect of ultrasonic power on the yield of HNTs. (Reprinted from Rong et al. (2016); with the permission of *Chemical Engineering Journal*)

have a significant impact on the biocompatibility of HNTs, e.g. copper oxide can cause damage to DNA; a purification step is essential, therefore. One way to remove metal oxides is to treat the HNTs with hydrochloric acid (Pasbakhsh et al., 2013). In HNTs the Al is exposed on the inner surface, in an octahedral gibbsite sheet (Al–OH); siloxane groups (Si–O–Si) are exposed on the outer surface, the water of the intermediate layer interacts with the surface groups Al–OH (Ouyang et al., 2018) (Fig. 2b). This configuration gives a positive charge to the internal lumen, consisting mainly of aluminum hydroxide, and a negative charge to the external surface, consisting mainly of silicon dioxide (Yah et al., 2012). In addition, compared to platelet-like clays (such as kaolinite and montmorillonite), an important advantage of HNTs is that such tubular clays do not need an exfoliation process and can be dispersed easily as single particles in polar polymers and water, forming stable dispersions in water and can be redispersed easily after sedimentation, making a good filler for biopolymers (Wu et al., 2019). Uncontaminated HNTs have a smaller zeta potential than pure silica particles. The HNTs, because of a negative zeta potential of ~–30 mV over a wide pH range (between 3 and 8), can form stable suspensions for up to 2–3 h (Katana et al., 2020). Furthermore, the external and internal charges of the HNTs are different, which allow selective immobilization of charged molecules (such as drugs, DNA, and proteins) on the outside or inside (Lvov et al., 2016). The immobilization on the internal lumens and on the external surface of the HNTs is driven by electrostatic adsorption. Following drug loading, especially negatively charged

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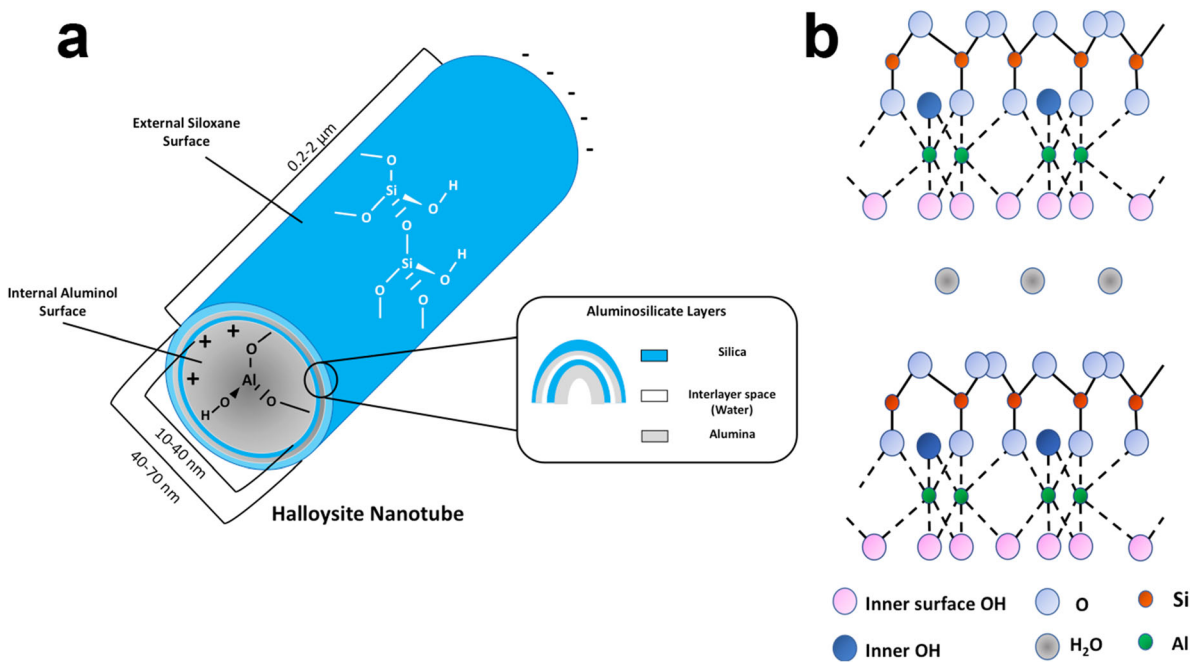


Fig. 2. **a** Structure of HNTs. **b** Crystal structure of HNTs

drugs, the lumen charge is neutralized and the zeta potential of the HNTs increases to ~–60 mV, with resulting colloidal formulations stable even for months (Lisuzzo et al., 2019a).

Several studies have shown a prolonged release of drugs, proteins, DNA, antiseptics, etc. for HNTs, with a water release profile of ~5–12 h. In addition, sealing the ends of the nanotubes or even incorporating the nanotubes loaded with the therapeutic agents into the bulk polymers allows an even slower release up to days, weeks, and even months (Lisuzzo et al., 2019b).

A classification of HNTs is based on their state of hydration. One group is represented by hydrated HNTs characterized by a crystal structure with a basal spacing of 10 Å; the second group is represented by dehydrated HNTs with a basal (d_{001}) spacing of 7 Å. After dehydration, the d_{001} spacing of the HNTs changes from 10 to 7 Å and this change is irreversible (Xia et al., 2019). Several characteristics make HNTs excellent candidates for various biomedical applications: a large surface area (up to 184.9 m^2/g) and a large pore volume (up to 0.353 cm^3/g) make HNTs effective as nano-platforms for the administration of therapeutic agents (Du et al., 2010). The size of the pores of the HNTs is in the range 2–50 nm, making it possible to refer to them as mesoporous materials (Joo et al., 2013). The main chemical and physical properties of HNTs are listed in Table 1.

HNTs are generally characterized using transmission electron microscopy (TEM) (Fig. 3a), scanning electron microscopy (SEM) (Fig. 3b,c,d), Fourier-transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD) (Hou and Wu, 2020). The presence of functional groups at the surfaces of the HNTs facilitates the loading of negatively charged biomacromolecules (such as DNA and RNA) at the lumen of the positive nanotube (Satish et al., 2019). The interaction between DNA and HNTs has also been exploited for the study of DNA damage through the use of HNTs-gold nanoparticle (AuNPs) and HNTs-silver nanoparticle (AgNPs) composites (Massaro et al., 2020). As seen, the HNTs are characterized by a negative surface charge on the outside and a positive charge on the internal lumen over a range of pH settings, allowing for various changes (Bretti et al., 2016). This characteristic, together with improved biocompatibility and reduced cytotoxicity, has allowed important progress in their applications in various fields, including biomedical sciences, i.e. the creation of platforms for the delivery of drugs and gene material, the development of scaffolds for tissue engineering, the production of medical devices for wounds, in the isolation of cancer cells, and for improved adhesion of human cells (Mantha et al., 2019).

The pore size of HNTs, on mesoporous scales, greatly exceeds that of many other synthetic porous materials, including carbon nanotubes. This property allows HNTs to be used in a wide range of applications, including application as a substrate on a nanoscale for the trapping of various functional molecules (Setter and Segal, 2020). The internal diameter of the HNTs allows not only the loading of small drug molecules but also of nucleic acids (DNA and RNA) and proteins (Shi et al., 2011). Two types of hydroxyl groups characterize HNTs, inside and outside, and can be used as active sites for functionalization and loading of drugs by modification (Bediako et al., 2018). Various classes and various types of drugs can be trapped in the internal lumen of the HNTs. In addition, various studies have confirmed that HNTs represent a new potential material for the administration of genetic material and anticancer drugs in the treatment of cancer, such as

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Table 1 Chemical and physical properties of HNTs

Empirical formula	$Al_2Si_2O_5(OH)_4{*}nH_2O$ (n = 0–2)
Length	0.2–2 μm
Outer diameter	40–70 nm
Inner diameter	10–40 nm
Aspect ratio (L/D)	10–
Pore diameter	2–50 nm
Surface	up to 184.9 m^2 /g
Pore volume	up to 0.353 cm^3 /g
Zeta potential (in a pH range of 3 to 8)	~ - 30 mV
Charge outer surface	Negative
Charge inner surface	Positive
Crystal structure spacing hydrated HNTs	d_{001} = 10 Å
Crystal structure spacing of dehydrated HNTs	d_{001} = 7 Å

curcumin and adriamycin, as reported by Liu et al. (2016), and for improved antitumoral action (Vikulina et al., 2020).

The characteristics of HNTs strengthen their role as promising nano-platforms for the trapping and controlled release of various therapeutic agents, including drugs, nucleic acids (DNA and RNA), peptides and proteins, agents with antibacterial (antifouling) action and biocide, and chemical agents such as polymers with self-healing and anticorrosive capabilities (Li et al., 2017a).

The HNTs have a number of disadvantages, however, including a tendency to form surface hydrogen bonds (with deterioration of the affinity in the formation of agglomerates or other materials) and polydispersity (Ma et al., 2018). Surface modification is required, therefore, to modify their surface in order to increase their hydrophilicity and biocompatibility. Modification of HNTs can be grouped into two main categories: (1) external modification can include the alkaline etching process, the grafting of organosilanes, NPs, surfactants, and

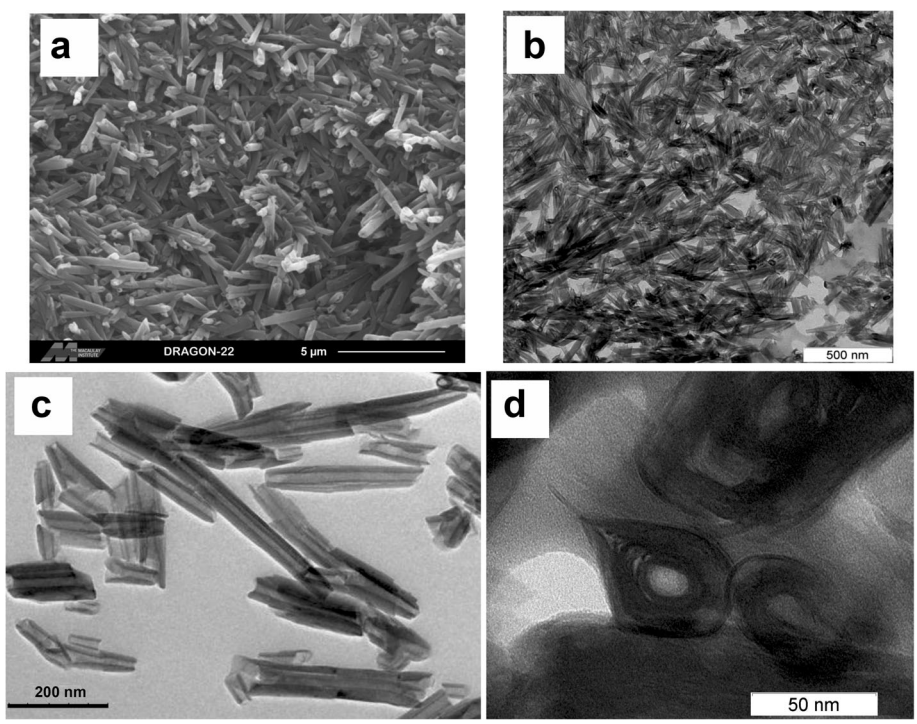


Fig. 3. a SEM image of HNTs. **b, c,** TEM images of HNTs, longitude-section. **d** TEM image of HNTs cross-section. (Reprinted from Vergaro et al. (2010); with the permission of *Biomacromolecules*)

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polymers on the external surface; and (2) while internal modification may include acid etching and the grafting of surfactants, biocomposites, polymers, and organosilanes on the internal surface (Tan et al., 2016). The modification of the HNTs increases their stability in corrosive environments, as well as conferring electrical and thermal properties. The alkaline and acid attack can cause a partial alteration of the shape of the HNTs, making them more compatible with various chemical agents (Joshi et al., 2013). In this regard, organosilanes can increase the versatility of HNTs, while surfactants can improve their stability, resulting in their greater dispersion in aqueous media. Furthermore, biomolecules can determine different properties, such as the manifestation of zwitterionic nature at different pH values and an increase in the mechanical and thermal properties of polymers (Lo Dico et al., 2018). Similarly, modification of the internal space of the HNTs can be achieved, allowing the controlled loading and release of various drugs (Venkatesh et al., 2019). Modification of the surfaces of the HNTs, moreover, plays a key role in their study, as it allows the use of HNTs in multiple environmental, catalytic, and, above all, biomedical applications.

Biocompatibility of HNTs (in vitro and in vivo studies)

One of the key characteristics in the use of HNTs as a nanobiomaterial in the treatment of cancer, as a system for the prolonged release of biologically active molecules, in medical, pharmaceutical, and personal care devices, is biocompatibility (Dionisi et al., 2016). The application of HNTs in biomedicine requires a scrupulous evaluation of the biocompatibility of this material. Recently conducted toxicity studies confirmed the significant biocompatibility of HNTs (Liu et al., 2012). The topical route, however, remains the most promising for application of HNTs, as this material is highly biocompatible but at the same time not biodegradable (Tully et al., 2016). Toxicity is often associated with synthetic materials. In this regard, the search for new, natural, green materials, such as nanoclays, is extremely promising (Rashid et al., 2021). Compared to carbon nanotubes, the main advantage of HNTs is represented by the fact that carbon-based materials typically require surface modification before their use in biomedical applications, while HNTs can be used directly, after washing and sterilization (Cho et al., 2020). Several studies have confirmed the biocompatibility of HNTs for a variety of cell cultures and microbial and animal models. Microbial cells (such as bacteria, yeast, and algae) and protozoa can tolerate HNTs at relatively high concentrations (1 mg/mL) (Fakhrullina et al., 2015). Various human cell lines have been treated with HNTs, either uncontaminated or surface-modified; the clay nanotubes have been determined to be non-toxic, and simple surface modification of the HNTs can attenuate their bioavailability and toxicity (Madani et al., 2013). For various types of human tissues and cells, HNTs are safe up to a concentration of 0.2 mg/mL, resulting in inorganic inclusions which are probably safe. Indeed, for freshwater protists, the safe concentration reaches 10 mg/mL (Massaro et al., 2018). In addition, the HNTs are apparently able to stimulate processes associated with cell proliferation and growth. Through the study of the differential expression of proteins, HNTs have been shown to improve the cellular response to injuries, infections, and irritations (Sandri et al., 2017). Finally, in vivo, the HNTs were found to be biocompatible for *Caenorhabditis elegans* nematodes. The toxicological profile of HNTs has been studied, mainly in vitro, with few in vivo studies (Kurczewska et al., 2017). A brief overview of the studies conducted on HNT toxicity in vitro and in vivo is given below.

In vitro Biocompatibility of HNTs

HNTs are a natural 'green' material with no associated significant biological hazards. In recent years, HNTs have been studied extensively in relation to possible use in various technological applications, including biomedicine (representing a cost-effective alternative to carbon and lipid nanotubes) (Cheng et al., 2020). Biocompatibility studies conducted on various cell lines (such as dermal fibroblasts and the epithelial adenocarcinoma cell line) support the biocompatibility of HNTs (Lai et al., 2013). Reduced cytotoxicity was also confirmed with studies conducted on animal cells and microbial cells. When HNTs are conjugated with iron oxide NPs (Fe_3O_4), the HNTs are able to reduce the cytotoxicity of iron oxide NPs by modifying their surface properties and suppressing their intrinsic cytotoxicity toward bacterial cells (Setter and Segal, 2020). Only a few studies have been conducted in vitro on tumor cell lines (e.g. HeLa, A549, MCF-7, and NIH-3T3) with the aim of evaluating the cytotoxicity of HNTs; all had promising results (Patil et al., 2015). Pure HNTs were found to be almost non-toxic to living organisms. HNTs, in the concentration range of 0.5–2.5 mg/mL, have no effect on the vitality and proliferation of yeast cells and bacterial cells (Rozhina et al., 2019). Higher concentrations led to minimal growth reduction which may be linked to mechanistic effects on cells, related to adsorption on cell walls or mechanical damage to the cell membrane (Huffer et al., 2011). Cell viability tests based on cell morphology and enzyme activity were used to show the viability of different types of cells treated with HNTs (Kamalieva et al., 2018). Surface modification of *Saccharomyces cerevisiae* (yeast) cells with polyelectrolyte and halloysite multilayers did not lead to toxic effects; ~99% of the cells coated with HNTs maintained their viability (Wang et al., 2014). Two human cell lines, MCF-7 (breast cancer cells) and HeLa (epithelial adenocarcinoma cells), were used to study the toxicity and cellular uptake of HNTs (Fig. 4) (Vergaro et al., 2010). A lung cancer cell line (A549) and a hepatoma cell line (Hep3B) were used to study the absorption and cytotoxicity of dextrin-coated HNTs. The coated HNTs showed reduced cytotoxicity toward human cell lines for concentrations not exceeding 100 μg/mL. The dextrin-modified HNTs penetrate mainly through the cell membrane of A549 cells and concentrate mainly in the vicinity of the nuclei, while for Hep3B cells, a low absorption rate and localization of the nanotubes at the level of the microvilli network has been detected (Naumenko and Fakhrullin, 2019). A greater level of cytotoxicity toward A459 cells (lung cancer cells) was demonstrated for the polyelectrolyte-modified HNTs compared with the unmodified nanoclay, which resulted in decreased cell viability, changes in nuclear morphology, and disruption of the

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cytoskeleton. The biocompatibility of a polymer/HNT composite material was evaluated; in particular, the HNTs were modified on the surface with a polycathione (such as poly (ethylenimine), poly (allylamine) or poly (diallyldimethylammonium)). To study the toxicity of polycathione-modified HNT, 2D and 3D cultures of human lung-cancer cells (A549) were used as models of cellular and nuclear changes (Tarasova et al., 2019). A recent study (Rozhina et al., 2020) evaluated the cytocompatibility of HNTs coated with octadecyl-trimethoxysilane (ODTMS). The study was conducted on human A549 lung cancer cells, revealing that HNT-ODTMS tubes are able to penetrate model cells without causing membrane damage. In addition, HNTs-ODTMS do not induce cellular apoptosis and do not cause an increase in the enzymatic activity of NO-synthase. The cytotoxicity mechanisms of HNTs have remained unclear thus far, however (Khodzhaeva et al., 2017). The study of protein expression on human colorectal adenocarcinoma cells and human colon epithelial cells (Caco-2/HT29-MTX) treated with co-cultured HNTs was performed by Lai et al. (2013); variations induced by exposure to HNTs, for 4081 proteins, were noted by the authors. Significant changes in protein expression were observed for cells treated with high concentrations of HNTs (~100 mg/mL) (Lai et al., 2013). Human umbilical vein endothelial cells (HUVEC) and MCF-7 cells exposed to HNTs at concentrations ≤200 μg/mL showed reduced cytotoxicity, good biocompatibility, and the ability to be absorbed by cells (Long et al., 2018b). Finally, the hemocompatibility of HNTs was studied for high concentrations of HNTs (up to 0.5 mg/mL); aggregation and changes in red blood cells (RBC) in PBS were observed. In addition, HNTs can determine the activation of the complement system and influence coagulation processes in a non-severe way (Wu et al., 2017). In another study (Can et al., 2021), the interaction with blood of HNTs modified with alkyl halides of various lengths, bromoethane (BrE), bromodecane (BrD), and bromooctadecane (BrOD), was evaluated. While HNT-BrE and HNT-BrD were shown to be non-hemolytic at a concentration of 1 mg/mL, HNT-BrOD slightly exceeded the hemolytic safety limit with induction of hemolysis of 6.6 ± 0.2%. The hemolytic ratio of the modified HNTs decreases with increase in the length of the alkyl chain of the halides, with a consequent significant antithrombogenic character (Can et al., 2021). The HNTs in one study were processed with anticoagulated rabbit blood to evaluate their hemocompatibility. The hemolysis test showed that hemolysis ratios were <0.5% lower, confirming a non-hemolytic effect of the HNTs (Liu et al., 2015c). Furthermore, the HNTs caused a reduction in the plasma recalcification time (in a dose-dependent manner), due to the activation of the platelets, showing procoagulant activity (Liu et al., 2015c). Further studies on the interaction of HNTs with blood components are needed in order to provide important guidance in terms of the design of HNT-based nanoformulations applied to clinical research and biomedicine.

In vivo Biocompatibility of HNTs

In vitro studies on the biocompatibility of HNTs have shown a reduced level of cytotoxicity; in vivo toxicity studies can provide important information on the mechanism of action of HNTs within the body, however (Liu et al., 2015a). Organisms of various evolutionary levels have been used to conduct a limited number of studies during the past 5 y. The protozoa, *Paramecium caudatum*, showed positive chemotaxis (~70%) toward HNTs (and other clay nanoparticles), unlike silica and bentonite which induced overall negative chemotaxis (~80%) at a concentration of 10 mg/mL, while graphene oxide rejected the protozoa even at a concentration of 1 mg/mL (Kryuchkova et al., 2016). Furthermore, the lowest reduction per cell in the number of food vacuoles was observed for HNTs. *Escherichia coli* cells modified with HNT/polyelectrolytes (nematode food source) were used to determine the effects of HNTs in free-living nematodes, such as *Caenorhabditis elegans*. The absorption of the HNTs did not affect the viability of the nematodes, with a localization of the nanotubes exclusively in the intestine, while the reproductive organs (spermatheca, ovaries, and uterus) were devoid of HNTs. In another study, the toxicity of HNT was evaluated using *C. elegans* as a model organism. The results obtained showed a negative effect of HNTs on the reproduction of *C. elegans* without affecting life span or other phenotypes, such as locomotion capacity, suggesting that HNTs do not have long-term toxic effects (Zhao et al., 2019a). These reduced toxic effects of HNTs compared to other nanoformulations (such as graphene oxide, TiO_2 NPs, and carbon nanotubes) can be explained by a relatively low absorption of the nanotubes by the intestinal cells and with an extremely limited transport to other districts (tissues and organs) of nematodes (Fakhrullina et al., 2015). The biocompatibility of HNTs in vivo was also tested, by analyzing the early embryonic development of zebrafish. No significant changes were observed in the survival rate of zebrafish larvae and embryos at different stages of development when exposed to HNTs (concentrations between 0.25 and 10 mg/mL) (Fig. 5). In addition, HNTs induce hatchability of zebrafish embryos and have no effect on morphological development for a concentration of ≤25 mg/mL. In this case the HNTs were also located mainly in the gastrointestinal tract of the zebrafish larvae (as for the nematodes) (Long et al., 2018b). For the toxicity of HNTs in vivo for mammals, only very few studies have been done. Among these, Wang et al. (2018) estimated the hepatic toxicity of pure HNTs administered orally in mice, observing that HNTs are able to stimulate their growth at low doses (5 mg/kg) without inducing liver toxicity. The HNTs inhibited the growth of the mice at half (50 mg/kg) and high (300 mg/kg) doses, however. For large doses of HNTs, hepatic aluminum accumulation and significant oxidative stress were observed, leading to hepatic dysfunction and histopathological changes. In addition, HNTs in mice are potential inducers of subchronic toxicity after inhalation. A block of autophagic processes with consequent accumulation of p62 was observed (with induction of apoptosis, oxidative stress, and inflammatory responses). An inversion of the results was observed following oral administration of trehalose to mice, with a reduction in p62 levels, which favored autophagy and led to lower toxicity related to HNTs (Ryman-Rasmussen et al., 2009). These results confirm that HNTs represent one of the safest clays for biomedical applications.

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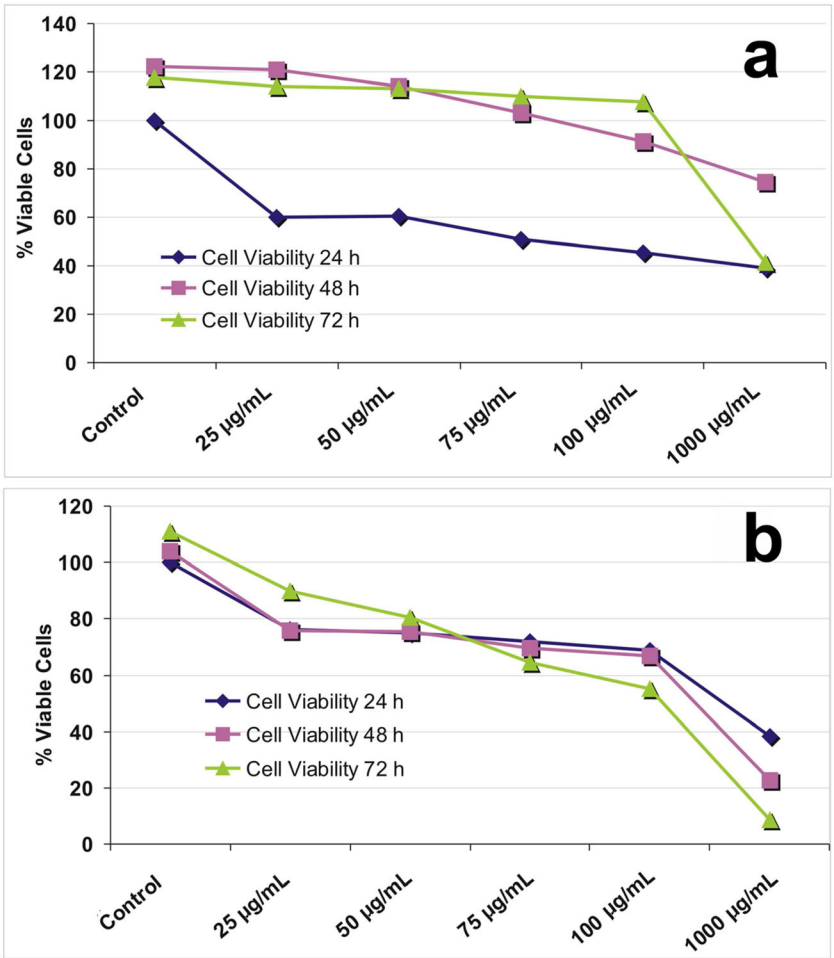


Fig. 4. Viability of **a** HeLa and **b** MCF-7 cells treated with increasing concentrations of HNTs for 24, 48, and 72 h. (Reprinted from Vergaro et al. (2010); with the permission of *Biomacromolecules*)

Applications of Halloysite Nanotubes in Biomedicine

HNTs have many biological and related applications. The present review aimed to list and discuss the main goals achieved by HNTs in biomedicine/nanomedicine in the recent past and the future prospects of this emerging nanomaterial in the biomedical sciences. The main applications of HNTs in clinical settings and in the development of new vectors for the delivery of therapeutic agents are highlighted here (Zhao et al., 2020). Several studies have focused on the trapping and controlled release of substances of clinical interest: drugs, including nifedipine, dexamethasone, furosemide, and resveratrol; biopharmaceutical agents including enzymes (lipase, laccase, and glucose oxidase) and nucleic acids; natural compounds (vitamins); and biosensors for imaging and contrast agents (Homayun and Choi, 2020). In addition, HNTs have also found application in tissue engineering and regenerative medicine with the development of targeted self-healing nanocomposites (for bone and dental cement, wound healing, and strengthening of microvascular networks) and tissue scaffolds. In addition, HNTs have found use in oral and topical drug administration (Bonifacio et al., 2017). The main applications of HNTs in biomedicine are summarized schematically in Fig. 6.

Drug Delivery

HNTs have been examined in many studies as nanosystems for drug delivery as well as for targeted drug delivery. Several strategies are used for the trapping of drugs within the lumen or on the surface of the HNTs, including intercalation, adsorption, and tubular trapping (vacuum strategy) (Hanif et al., 2016). Usually, the HNTs used as vectors for the administration of drugs first of all undergo modifications, which affect both the external surface and the lumen. This is a consequence of the fact that, very often, natural HNTs show weak interaction with drugs, not allowing prolonged release (Pan et al., 2017). In a study by Abdullayev and Lvov (2011), natural HNTs were employed as nuclei for layer-by-layer (LbL) entrapment; an increase in payload and continuous drug release for up to 100 h were noted, with the formation of caps at the end of the nanotube which helped to extend drug-release time. Natural

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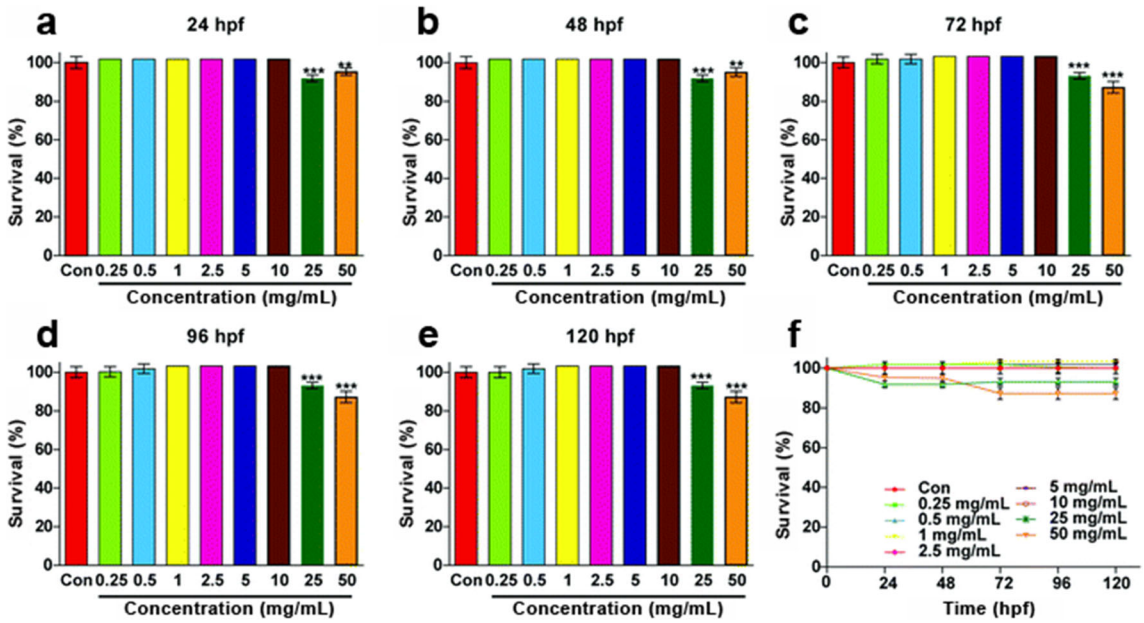


Fig. 5. Acute toxicities of HNTs in zebrafish. The survival rate was evaluated following treatment with various concentrations of HNTs at various time intervals: **a** 24 h, **b** 48 h, **c** 72 h, **d** 96 h, and **e** 120 h. (Reprinted from Long et al. (2018b); with the permission of the *Royal Society of Chemistry*)

HNTs were used to design a biocompatible vector for the administration of rabeprazole sodium (RAB) in order to counteract the acid degradation of this drug in the stomach. The results showed improved bioavailability and prolonged drug release (Khatoon et al., 2020). Generally, before the trapping of drugs on HNTs, the latter are subjected to various surface-modification strategies. One of these strategies involves modification of the surface of the HNTs with APTES (3-(aminopropyl)triethoxysilane) which is an organosilane, and this approach is a renowned functionalization method for both ease of use and reduced toxicity. Organosilane acts as an intermediate medium for binding the desired molecules. With APTES, silanol groups are introduced that can bind (through hydrogen bonding) to the hydroxyl groups present on the surface of the HNTs (Tian et al., 2015). APTES-functionalized HNTs were employed as aspirin transporters;

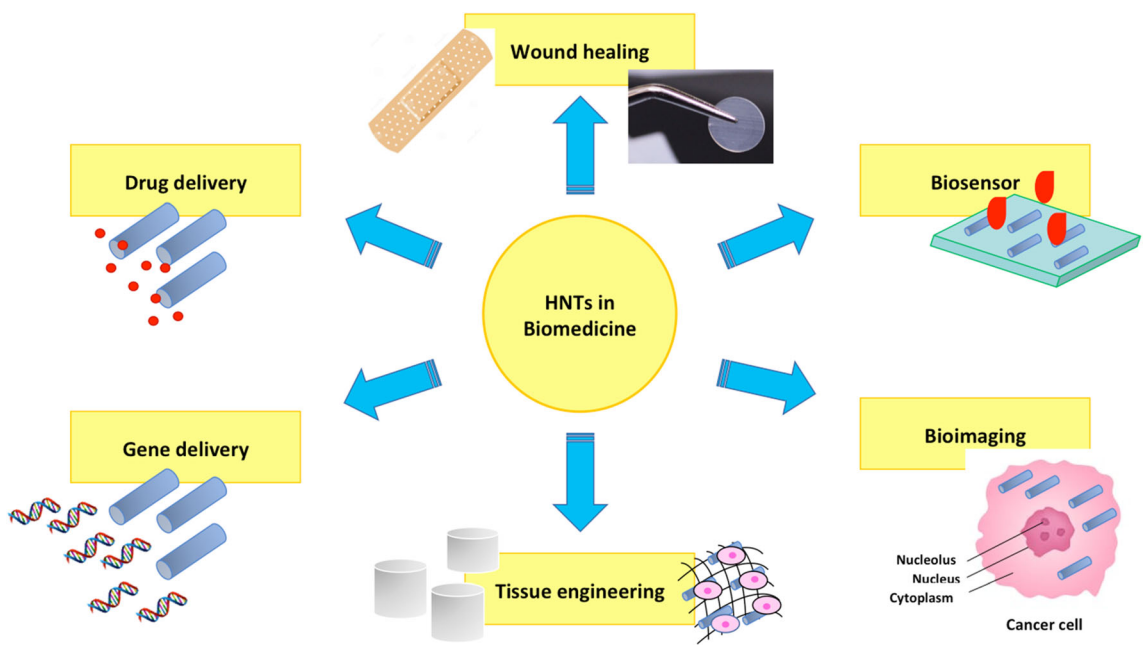


Fig. 6. Clinical applications of HNTs

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the results obtained showed an increase in loading efficiency, with an 11.8% weight load of aspirin, compared to 3.84% weight without functionalization (Lun et al., 2014). The release profiles of ibuprofen (IBU) loaded in both APTES-modified HNT (APTES-HNT) and unmodified HNT were investigated. The results revealed greater loading efficiency and a better release profile for APTES-HNT (Tan et al., 2014). In another study, chitosan-modified HNTs (now an emerging drug-delivery microsphere) were loaded with aspirin and the drug-release profile was investigated. The trapping efficiency of aspirin was 42.4% by weight for porous microspheres, ~20 times greater than that of uncontaminated HNTs (2.1% by weight) (Li et al., 2016). Furthermore, HNTs demonstrated a high trapping efficiency of camptothecin, while the results for an in vitro release study confirmed a much higher outflow of camptothecin from the nanosystem at pH 5 than that detected for higher pH values (6, 8, and 7.4). This study highlighted a pH-dependent release, which is vital considering that the pH of the tumor microenvironment in lysosomes and endosomes is acidic. For toxicity assessment, analyses of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) showed that the HNT loaded with camptothecin showed greater inhibition of cell growth against colon cancer cells (Dramou et al., 2018). A recent study by Sharif et al. (2019) showed microcomposites coated with chitosan for long-term drug release. The microcomposites produced showed a size of 0.151 ± 0.49 μm. Metoclopramide hydrochloride was used as a model drug, with drug release ranging from 35.14 ± 1.5 to 53.97 ± 5.23%. Furthermore, these microcomposites (chitosan-coated HNTs) have been shown to be pH responsive, with a 25 h release of 66.8% and 46.7% at pH 1.2 and 5.5, respectively. It follows that these microcomposites can be used as carriers for the long-term release of drugs in the treatment of cancer (Sharif et al., 2019). The functionalization of HNTs with an opposite matrix is a technique that has recently been used widely for the realization of a system for a controlled release of drugs. In another work, HNTs were integrated into electrospun poly-and-caprolactone (PCL) scaffolds with the aim of improving structural coherence. The HNT inserted into the scaffold acted as a nanocarrier for various antibacterial agents such as amoxicillin, brilliant green, chlorohexidine, doxycycline, iodine, gentamicin sulfate, and potassium calvulanate. These nanocomposites were able to inhibit bacterial growth for up to one month, showing promising potential in applications for sutures and surgical dressings (Lepoittevin et al., 2002). A potential selective antimicrobial formulation was developed by Fakhrullina et al. (2019) for pathogenic microflora, based on curcumin-loaded HNTs and externally coated with dextrin (DX); its efficacy was tested in *Caenorhabditis elegans* (nematodes). In vitro HNT+Curc/DX was able to inhibit effectively the growth of *Serratia marcescens* enterobacteria. In addition, in vivo treatment of *S. marcescens* infected nematodes with HNTs+Cur/DX resulted in improved fertility and restoration of longevity of *C. elegans* (Fakhrullina et al., 2019). Curcumin (thermosensitive) release kinetics studies were conducted for HNTs modified with biocompatible poly (N-isopropylacrylamide). The in vitro tests were aimed at simulating the gastrointestinal transit of the HNT nanoformulation loaded with curcumin (natural anticancer molecule). The results demonstrated a targeted release of the active molecule in the intestine (Cavallaro et al., 2015). Doxorubicin (DOX), another anticancer drug, has been encapsulated in multifunctional HNTs, and prolonged and targeted release of the drug has been investigated. In vitro studies of non-targeted and targeted HNTs revealed that targeted HNTs were able to accelerate tumor cell death by apoptosis. In addition, the in vivo studies have reinforced the fact that the targeted HNTs have no side effects in tumor-bearing mice, unlike free DOX which has induced tissue damage previously (Yang et al., 2016). To obtain an improved loading and a prolonged release of quercetin (a drug with reduced solubility in water), HNTs with grafted (polyethylene glycol)-amine (PEG) functionalized with carbon dots (to confer fluorescent properties) were made. Quercetin has antioxidant properties, and the goal was to facilitate targeting of tumor tissues. To improve targeting and enhance cellular uptake, biotin was conjugated to the free amine groups of the PEG (Yamina et al., 2018). A more recent strategy that has shown very promising results in drug delivery involves functionalization with dendrimer. In a study by Kurczewska et al., (2018), the synthesis of functionalized HNTs with polyamide dendrimers (PAMAM) and 3-aminopropyltrimethoxysilane for the administration of drugs such as chlorogenic acid, salicylic acid, and IBU (as model drugs) was demonstrated. The dendrimer functionalized HNT revealed a better entrapment capacity for the three drugs compared to unmodified HNTs and APTES-HNTs. In addition, a very slow release rate was reported for chlorogenic acid and salicylic acid from dendrimer-modified HNTs while the release of IBU was similar to that recorded for nanotubes functionalized with APTES (Fig. 7). Because in vivo biocompatibility studies have not shown damage to living organisms by HNTs functionalized with dendrimers, it follows that the main advantage of functionalization of HNTs with dendrimer is the improved biocompatibility, resulting in the promising hybrid nanotubes for use in biomedical applications (Kurczewska et al., 2018).

In conclusion, HNTs, both in pure and modified form (e.g. compressed with polymers), represent promising natural nanosystems for the trapping and controlled and prolonged release of drugs, both with reduced water solubility (such as aspirin, curcumin, IBU, and DS) and normal water solubility (such as amoxicillin), with satisfactory results, underlining the versatility of HNTs as nanocontainers for a large variety of drugs. The applications of HNTs in drug delivery with relative advantages are summarized in Table 2.

Vaccine and Gene Delivery

Recently, due to advantageous properties such as reduced cytotoxicity (for bacterial and human cells), good biocompatibility, and remarkable mechanical strength, HNTs are emerging as potential non-viral nanosystems for the delivery of vaccines and genes (Guo et al., 2012). In one study, HNTs together with multi-walled carbon nanotubes functionalized with carboxylic groups (COOH-MWCNT) were used in the analysis of antigen release in order to improve the immune response to a recombinant LipL32 protein (rLipL32). The

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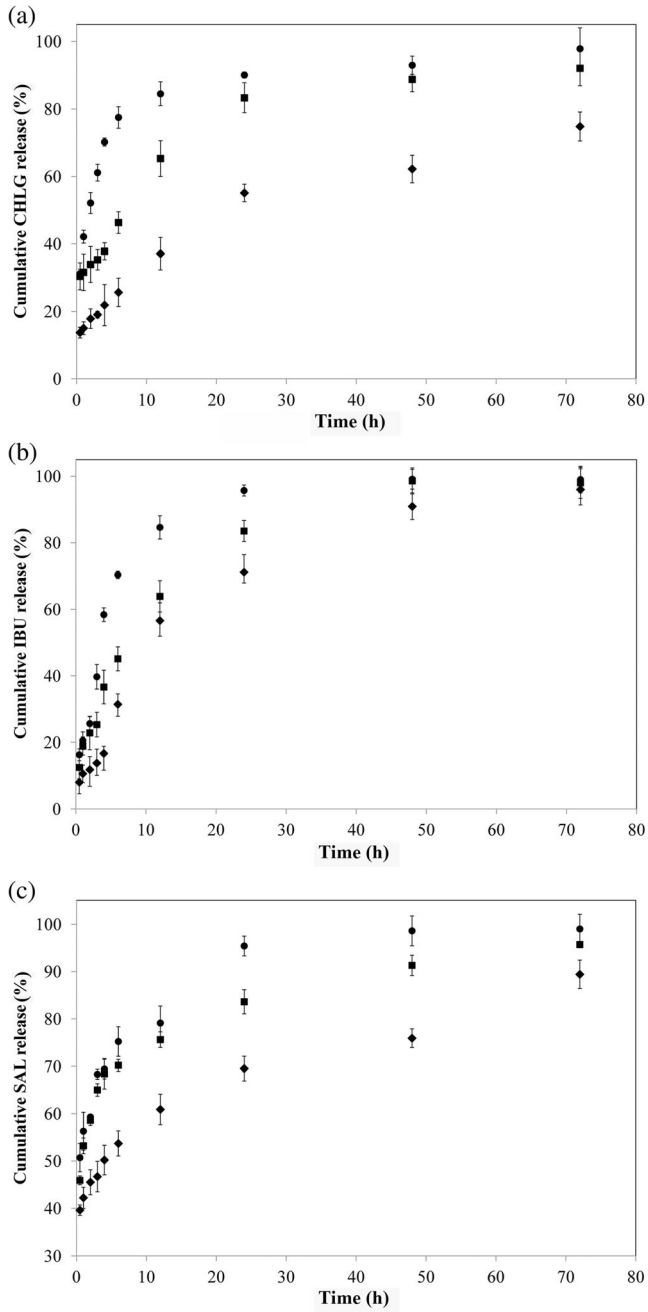


Fig. 7. Release kinetics of **a** CHLG, **b** IBU, and **c** SAL by: ● HNT, ■ APTES-HNT, and ♦ PAMAM-HNT. (Reprinted from Kurczewska et al. (2018); with the permission of *Applied Clay Science*)

results obtained showed that immunization by HNTs and COOH-MWCNT increased significantly the IgG antibody titer specific to rLipL32 in golden Syrian hamsters with leptospirosis disease (Teixeira et al., 2019). With regard to gene therapy, several disadvantages are associated with the use of viral vectors; so, increasing attention is paid to new non-viral nanocarriers for the release of genes. APTES-functionalized HNTs were used by Zhang et al. (2019) as vectors for the administration of gene material, and in particular the antisense oligodeoxineuclotide as a therapeutic gene was bound on the outer surface of functionalized HNTs with the aim of targeting survivin (a protein belonging to the family of apoptosis inhibitors) (Shi et al., 2011). In another study (Long et al., 2018a), modification of HNTs with polyethyleneimine (PEI) was shown to be effective at the release of plasmid DNA (pDNA) and short interference RNA (siRNA). These PEI-modified HNTs were employed for the intracellular delivery of antisurvivine therapeutic siRNA. Western blot analysis

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Table 2 HNTs in drug delivery

Nanoformulation	Drug	EE* (%)	LC*	Fraction released	Model	Advantages	Reference
Natural halloysite nanotubes (HNTs) modified with 3-aminopropyltriethoxysilane (APTES)	Aspirin	11.98	–	68% by weight of aspirin released within the first hour, and the release stops for 600 min	–	improved drug encapsulation	Lun et al. (2014)
Poly(N-isopropylacrylamide)-halloysite Nanotubes	Curcumin	3.6	–	in acid solution no release was observed in the first 50 min; after this time a release of 2.5% in acid solution and a release of 10% at pH 6.8 was observed	simulated gastric fluids and simulated intestinal fluids	prevents the degradation of curcumin in an acid environment, biocompatibility and heat responsiveness	Cavallaro et al. (2015)
Sodium alginate/hydroxylapatite/halloysite nanotubes (SA/HA/HNTs)	Diclofenac sodium (DS)	74.63±1.65	23.35± 0.05%	9.19 mg g^{-1} h^{-1}	–	improved loading and prolonged release of the drug	Fan et al. (2013)
Functionalized HNT (pH and GSH-responsive)	Curcumin	2.9	_	25% curcumin released within 100 min, in a buffer containing 10 mM of GSH (~5% curcumin released in the absence of GSH), followed by sustained release	Hep3B and HA22T/VGH (poorly differentiated hepatoma cell lines)	improved stability under physiological conditions, controlled prodrug release and the prodrug HNT-Cur compared to the free Cur induces high cytotoxicity	Massaro et al. (2016)
APTES-modified Hal	Ibuprofen (IBU)	14.8	–	–	–	prolonged drug release due to strong affinity based on electrostatic interaction	Tan et al. (2014)
APTES-HNT	Ciprofloxacin (CIP)	70 ± 1.7	70.16 ± 0.5 mg/g	in PBS 92% ± 3% in water 60% ± 2% in 9 h of study	_	prolonged release of the drug and improved bioavailability of the drug after administration	Rawtani et al. (2017)
HNTs/PCL nanocomposites	Amoxicillin, Brilliant Green, Chlorhexidine, Doxycycline, Gentamicin sulfate, iodine and Potassium calvulanate	–	–	99.95% Brilliant Green is released in 1.1 days, an overall sum of 91.67% Amoxicillin and Potassium calvulanate is released in 9.6 days	–	sustained release profile for antibacterial agents and inhibition of bacterial growth for up to one month	Patel et al. (2016)
PMMM coated halloysite	Paclitaxel	_	7.5 ± 0.5%	40% in 120 h	Human cervical epithelial cancer cells (HeLa) and human lung adenocarcinoma cells (A549)	sustained release, higher release at basic pH and significant antitumor action of	Yendluri et al. (2017)

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Table 2 (continued)

Nanoformulation	Drug	EE* (%)	LC*	Fraction released	Model	Advantages	Reference
						halloysite-paclitaxel composites	
Folic acid-conjugated chitosan oligosaccharide- magnetic HNTs (FA-COS/MHNTs)	Camptothecin	_	227.10 mg g^{-1}	sustained drug release up to 60 h	Human epithelial colorectal adenocarcinoma cells (Caco- 2)	cell growth inhibition and specificity in targeting cancer cells	Dramou et al. (2018)
PEG amine-grafted HNT (HNTs-g-PEG)	Quercetin (Que)	_	278.36 mg g^{-1}	in 7 days 44.20% of Que is released at pH 7.4, while at pH 6.8 and 5.0 67.92% and 75.43%, respectively, of Que is released	_	improved drug loading	Yamina et al. (2018)
HNTs-S-S-β-CD-Ad-PEG-FA	Doxorubicin	14.2%	_	55% of DOX and 70% of DOX is released in the first 10 h, in DTT 5 mM and DTT 10 mM, respectively	SKOV3 and 293T cells	Folate-mediated targeting, oxidation-sensitive drug release, and accelerated apoptosis of cancer cells	Hu et al. (2017)
PAMAM-functionalized halloysite nanotubes	Chlorogenic acid, ibuprofen and salicylic acid	_	123.16 mg/g (chlorogenic acid), 182.72 mg/g (ibuprofen) and 39.52 mg/g (salicylic acid)	_	pure cultures of Acutodesmus acuminatus (Lag.) Tsarenko (syn. Scenedesmus acuminatus (Lag.) Chodat)	controlled release of drugs and not toxic to live organisms	Kurczewska et al. (2018)
Chitosan oligosaccharide-grafted HNTs (HNTs-g-COS)	Doxorubicin (DOX)	55.5 ± 3.8%	2.63 ± 0.14%	in PBS (pH 7.4), 6.40% of DOX is released in 45 h, while in cell lysate (slightly acidic tumor environment) 61.9% of DOX is released in 12 h	MCF-7 cells and 4T1-bearing mice	increased apoptosis of MCF-7 cells and tumor inhibition ratio of DOX @ HNTs-g-COS by 83.5%, while it was 46.1% for free DOX	Yang et al. (2016)
Chitosan-coated halloysite nanotubes loaded with curcumin-Au hybrid nanoparticles	Curcumin	12%	_	at pH 5.5, 95% of CUR is released in 48 h, while at pH 7.4, 10% of CUR is released in the same period	MCF-7 cells	effective anti-tumor action in the intracellular environment of tumor cells (pH 5.5) compared to the extracellular environment (pH 7.4)	Rao et al. (2018)
Positively charged halloysite nanotubes functionalized with triazolium salts (f-HNT)	Curcumin and triazole	12.5%	_	at pH 1 the release of curcumin reaches a plateau in 150 min	HA22T/VGH, HepG2 and Hep3B is a poorly differentiated hepatoma cell line	the transport system improves the solubility of curcumin in water and cytotoxic action of f-HNT loaded with the drug towards different cell lines	Riela et al. (2014)

**EE* Encapsulation efficiency, *LC* Load capacity

confirmed that siRNA administration via PEI-HNTs reduced target protein levels in PANC-1 cells with significant reduction in survivin gene expression, reinforcing its therapeutic potential in cancer treatment (Long et al., 2018a). In the treatment of genetic diseases and cancer, gene therapy has shown considerable potential; gene-transport systems must meet extremely important requirements, however, such as biocompatibility and cytotoxicity, which continue to hinder this treatment strategy (Christensen et al., 2019). HNTs have shown promising results for application as nanocarriers in gene therapy due to their easy availability, significant biocompatibility, remarkable mechanical strength, and various structural advantages.

Cancer Therapy and Cancer and Stem-Cell Isolation

Several strategies have been examined for the targeted delivery of anticancer drugs via HNTs. In a study by Mirzapur et al. (2018), HNTs were loaded with resveratrol and drug delivery to cancer cells was investigated. The viability study with MTT assay was carried out using a tumor cell line model (MCF-7) which showed that the nanotubes loaded with resveratrol were able to increase cytotoxic action significantly, resulting in cell death by apoptosis. In another study, multicomponent HNT (FA-Fe_3O_4 HNT) was analyzed as a targeted delivery system of DOX (anticancer drug) in cancer cells. The results confirmed that the drug loaded in FA-Fe_3O_4 HNT is able to induce apoptosis of HeLa cells (model cells) (Luo et al., 2020a). Curcumin is a natural polyphenol with antioxidant properties that has found application because of its potential anticancer activity; several strategies have been used for loading it into HNTs (Abdullayev et al., 2009). In one of these, positively charged HNTs were functionalized with trizolium salts. This HNT-based delivery system was employed for the delivery of curcumin in several cell lines. HNTs functionalized with curcumin-loaded trizolium salts were shown to be active in several cancer cells (Massaro et al., 2020). In another work, by Massaro et al. (2020), biopolymer-modified HNTs were used for the targeted delivery of anticancer drugs. In particular, HNTs modified with chitosan (HNT-g-CS) were studied for the administration of curcumin in cancer cells. Curcumin-loaded HNT-g-CSs showed cytotoxicity toward various tumor cell lines, such as HeLa, HepG2, SV-HUC-1, MCF-7, EJ, and Caski; among these the EJ cell line showed an increase in the apoptotic process. In addition, the amount of reactive oxidative species (ROS) induced by curcumin encapsulated in HNT-g-CS is greater than that of non-encapsulated curcumin, making this platform a promising system for the administration of anticancer drugs (Massaro et al., 2020). HNTs modified with chitosan oligosaccharide have been employed for the release of another anticancer drug, DOX, for the treatment of breast cancer using the MCF-7 cell line. DOX-loaded HNT-g-CS was internalized by MCF-7 cells, triggering mitochondrial damage and attacking nuclei (Yang et al., 2016). In another study, by Li et al. (2017a), the release efficacy of anticancer drugs by modified HNTs was tested, with a new-design nanotube-microsphere, in which the HNTs are enclosed in a polymeric shell (hydroxypropylmethylcellulose acetate succinate) sensitive to pH. Atorvastatin and celecoxib were used as model drugs, as they have different physicochemical properties, and demonstrated colon cancer inhibition. In that study, the pH-reactive polymer/HNT composite system hindered premature drug release at pH 6.5, allowing for quicker release and increased drug permeability at pH 7.4 (Li et al., 2017a).

An important advance in the diagnosis and treatment of cancer is linked to the possibility of capturing and isolating rare circulating cancer cells from the blood of patients, for the development of personalized therapies. With this in mind, HNT surfaces were coated with a layer of poly-1-lysine and functionalized through a selective recombinant human protein. This strategy resulted in an increase in the capture capacity of leukemic cells (under flow) by HNTs (Saif and Asif, 2015). In another study, a rapid, effective, and economical manufacturing procedure was employed to coat large, raw HNTs by thermal spraying of HNT dispersions in ethanol. All this led to improved surface communication between the HNTs' coating and tumor cells, with effective capture of the tumor cells compared to normal cells (except for HeLa cells). Coated HNTs have also been shown to be efficient in capturing cancer cells in patient blood samples (with metastatic breast cancer) and in artificial blood (He et al., 2018). This effective ability to capture tumor cells constitutes a potential for early diagnosis and offers an important window into the progression and feasibility of personalized anti-cancer therapy. In addition to recent consideration of the potential of HNTs in the capture and isolation of cancer cells, their use in the isolation of stem cells was also considered. Research (Luo et al., 2020b) has shown that a three-dimensionally printed polylactic acid (PLA) pattern functionalized with a layer of polydopamine helped to bind the HNTs effectively on the surfaces of the PLA pattern in order to orient the guided cells. The HNTs improved significantly the hydrophilicity and roughness of the PLA pattern, and in vitro studies with human mesenchymal stem-cell cultures confirmed that the PLA pattern with HNTs has a different ability to induce cell orientation based on different widths of the strips (Luo et al., 2020b). Antitumor therapy and the study of stem cells are an important and delicate field of research in biomedicine and the use of HNTs widely available in nature could represent an important turning point, but with many points yet to be explored.

Tissue Engineering

HNTs have been used in the development of nanocomposites as supportive matrices for the controlled release of various drugs for various targets, thus finding a role as a delivery system in tissue engineering (Schmitt et al., 2015). An example is the incorporation of alkaline phosphatase (ALP) in HNTs in bone repair. In this system, the HNT acted as a heat sink resulting in an increase in the thermal stability of the ALP. In addition, a significant improvement in the activity of the enzyme (ALP) was recorded, with the promotion of the biomineralization process, which was studied in vitro with the use of calcium glycerophosphate as a substrate. This bioactive nanocomposite could also be incorporated into biomaterials applied as scaffolds in tissue engineering (Liu et al., 2012; Satish et al., 2019).

In tissue engineering, the creation of a scaffold, sufficiently suitable and competent in supporting the three-dimensional

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formation of tissues, is essential. These scaffolds should be able to meet specific basic requirements such as: (1) an adequate pore size (in order to facilitate nutrient diffusion and seeding) with high porosity; (2) lower rate of degradation and biodegradability (the scaffold must be absorbed by the surrounding tissue and at the same time favor the formation of new tissue); and (3) high mechanical strength for a suitable support for the growth of the new tissue (Chan and Leong, 2008). HNTs aligned in parallel strips on a solid support represent a promising platform capable of promoting the proliferation of human bone mesenchymal stem cells (HBMSC), promoting osteogenic differentiation in the absence of growth factors (Zhao et al., 2019b).

The use of HNTs in tissue engineering is a very recent challenge, however; only a few promising studies have been carried out over the past decade. The basic idea is to couple HNTs with various compounds with the aim of creating potential folds capable of promoting the growth of bones and tissues (Naumenko et al., 2016). Chitosan-HNT nanocomposite scaffolds are characterized by high compressive strength, Young's modulus, and significantly improved thermal stability compared to pure chitosan scaffolds. Although the HNTs particularly affected the porosity and porous structure of the scaffolds, they did not induce any toxicity on the cells. In addition, the cells were reported to adhere to and to develop optimally on the scaffold. Chitosan-HNTs produced by electrospinning, with 0, 2, and 5 wt.% HNT, led to nanocomposites that demonstrated increased tensile strength and increased Young's modulus with increasing HNT concentration. The chitosan-HNTs represent ideal membranes for bone-tissue engineering due to their thermal stability and improved mechanical properties (Liu et al., 2013). Another compound coupled with the HNTs (to make scaffolds) is alginate, which demonstrates reduced water absorption, high compressive strength, and reduced degradation rate. The HNTs also resulted in an improvement in the thermal stability of the alginate scaffolds, in addition to an increase in cell adhesion and improved biocompatibility. In one study, the incorporation of HNT made it possible to improve the physical properties of a sodium alginate scaffold and the composite was cross-linked with calcium ions. With low loading of HNTs, the composite scaffold demonstrated increased cell adhesion and proliferation in cultured preosteoblasts (MC3T3-E1), useful in bone-tissue engineering (Liu et al., 2015b). In one paper (Qi et al., 2010), metronidazole-loaded HNT grafted into poly (caprolactone)/gelatin microfibers through electrospinning as a membrane for sustained drug release and guided bone regeneration was developed. Once again, scaffolds of poly (L-lactic acid) nanofibers were reinforced with the use of unidirectionally aligned HNTs in order to increase Young's modulus, tensile strength, and fracture stress. A porous antibacterial membrane for bone regeneration was produced, with multilayer polylactic acid (PLA)/HNT encapsulated with gentamicin (an aminoglycoside antibiotic). The studies carried out have shown a satisfactory antibacterial efficacy against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria, resulting in a promising tool in the prevention of infections during bone regeneration (Qi et al., 2010; Berton et al., 2020). In one study (Utech and Boccaccini, 2016), a composite chitosan-gelatin-agarose hydrogel grafted to HNT demonstrated increased water absorption and improved mechanical strength and thermal properties. These scaffolds implanted in rats showed excellent resorption even at 6 weeks. Furthermore, the newly formed connective tissue located in the vicinity of the scaffold showed the complete restoration of blood flow thanks to neovascularization (Utech and Boccaccini, 2016). In another work in gellan rubber matrices, HNTs were integrated with the aim of creating composite hydrogels with modular physical properties. These systems showed good biocompatibility with human dermal fibroblasts, when the cells were seeded on the surface of the gels or encapsulated within the composite matrix. In addition, the fibroblasts seeded on the hydrogel with HNT showed high metabolic activity due to the improved mechanical and topographic characteristics (Venugopal and Ramakrishna, 2005; Alkatheeri et al., 2015). These scaffolds, therefore, prove suitable in soft-tissue engineering applications (such as liver, pancreas, chondral, and skin regeneration). The group of Huang et al., 2017 incorporated the HNTs in a methacrylate gelatin hydrogel in order to evaluate the potential of the composite hydrogel to promote bone-tissue regeneration. In vitro hDPSC cells (human dental pulp stem cells) incubated with the hydrogel revealed increased expression of genes related to the osteogenesis process and subsequently improved in vivo tissue-regeneration rates in rats with cranial defects. Though few studies on the role of HNTs as bionanocomposites in tissue engineering are found, the current results confirm an important potential of HNTs in tissue engineering (Ou et al., 2020). Overall, these studies demonstrated that the incorporation of HNTs into agarose, chitosan and gelatin bioscaffolds provides structural stability, improved adhesion, and cell growth. In vivo studies on rats showed the ability to promote neoangiogenesis in the vicinity of the scaffold without inducing an inflammatory reaction. In addition to the use of HNTs to supplement scaffolds based on alginate, polycaprolactone and gellan gum, in which HNT silicate ions have been used to stimulate osteoblasts in collagen secretion.

Wound Healing

HNTs are characterized by high mechanical strength, good biocompatibility, and hemostatic properties which make them suitable for applications in the development of medical devices. In addition, several studies have evaluated the possible use of HNTs as biocompatible nano-platforms in the controlled and prolonged release of antiseptic and antibacterial drugs, for application in wound healing (De Silva et al., 2018). In a study by Patel et al. (2016), copper-benzotriazole-coated HNTs were loaded with an antiseptic drug, Bright Green, and the release kinetics were investigated, detecting sustained release, over the period 50 to 200 h, from the nanotube. In addition, controlled release of iodine and amoxicillin by HNT took place (Patel et al., 2016). In another study by Huang et al. (2017) flexible three-dimensional chitosan composite sponges were manufactured with the addition of HNT, with improved

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toughness, compressive strength, and elastic modulus. The integration of HNTs resulted in an improvement in the blood coagulation capacity of chitosan. In vivo studies demonstrated the biocompatibility and improved the wound-healing properties of composite sponges, with improved skin reorganization and re-epithelialization compared to HNT or chitosan used separately. All this is linked to the induction of the activity of repairing inflammatory cells, stimulated by the prolonged release of chitosan oligosaccharides (homo- or hetero-oligomers of N-acetylglucosamine and D-glucosamine) making this nanocomposite a promising medical device in wound repair. (Huang et al., 2017). In another study, HNTs were used to deliver vancomycin in an alginate-based medical device. The resulting dressing exhibited high stability and neutrality in relation to living organisms used in biological tests, resulting in a potential parameter-optimized dressing for effective long-term wound treatment (Satish et al., 2019). In a similar study by Xue et al. (2015), ciprofloxacin (antibiotic) was dispersed in a gelatin-based matrix in which the HNTs loaded with polymyxin B sulfate (antibiotic) were dispersed uniformly. The combined use of the two antibiotics within the nanocomposite demonstrated synergistic antimicrobial activity. In addition, this bio-nanomaterial has been shown to have effective properties such as significant water absorption, reduced toxicity, regulation of biodegradation, and high elasticity, optimal characteristics for applications in wound healing (Xue et al., 2015).

The grafting of the HNTs has also made it possible to strengthen an elastic nano-fibrous material formed by PCL and gelatin, for the development of a device that could be used in wound dressing with a prolonged drug release. The drug was administered using HNTs modified with silane and loaded with metronidazole; these were inserted into electrospun polycaprolactone/gelatin microfibers, creating membranes for guided tissue regeneration, which contained ~25 wt.% metronidazole. These membranes were biocompatible and able to inhibit the internal growth of fibroblasts, as well as colonization by the anaerobic *Fusobacterium nucleatum* thanks to the prolonged release of metronidazole, up to 20 days (Shi et al., 2018). In addition, the HNTs have been integrated into a wound-healing powder formulation. First of all, a nanocomposite based on HNTs and chitosan oligosaccharide was created. The resulting powder was applied to burns on the backs of rats for 7 days, with evident improvement in the healing process (Sandri et al., 2017). HNTs loaded with antibiotics and incorporated into different types of biopolymer matrices demonstrate the extraordinary potential for wound-dressing applications with integrated anti-inflammatory and antimicrobial activities.

Biosensing

Interest is growing in the application of nanoformulations as potential future contrast agents (CAs) in molecular ultrasound imaging, especially if functionalized on the surface with specific biological recognition elements (e.g. ligands) for targeted delivery and release of therapies (Andreou et al., 2017). Nanomaterials have numerous advantages that make them useful as CAs including their optical and magnetic properties that can be modified and adapted by manipulating their composition, size, structure, and shape (Kumar et al., 2011). Parametric evaluation of the efficiency of HNTs as a scatterer in safe ultrasound-based molecular imaging has been demonstrated in a published work by Soloperto et al. (2013). The results obtained confirmed the possible use of a clinically readily available ultrasound system for the detection of ultrasonic backscatter generated by different concentrations of HNTs using sonication frequencies of 5.7 and 7 MHz (Soloperto et al., 2013). The key to creating a highly sensitive electrochemical enzymatic biosensor is to be able to obtain a direct transfer of electrons between the electrode surface and the enzyme, and this can be achieved through an improved immobilization of the enzyme with high load capacity (Grieshaber et al., 2008). In one paper, by Cheng et al. (2020) HNTs were used in enzymatic immobilization for glucose detection. A hybrid nanocomposite consisting of silver nanoparticles, with an average diameter of 10 nm, was immobilized on the surface of HNTs, modified with silane obtained by in situ chemical reduction of silver ions (Ag^{+}). The hybrid nanocomposite thus produced constituted a platform for the immobilization and electrical wiring of the redox enzyme, glucose oxidase (GO*x*), which catalyzes the oxidation of glucose into D-gluconolactone with the production of H_2O_2. The immobilization of the enzyme resulted in the improvement of the direct transfer of electrons with the formation of a highly sensitive and stable device for the detection of glucose in the monitoring of diabetic patients (Cheng et al., 2020). In another study, a nanocomposite based on HNTs functionalized with polyaniline (PANI) was used as an extremely sensitive biosensor for the detection of ascorbic acid (AA). A composite sensitivity to AA of ~ 826.53 mA mM^{-1} cm^{-2} over a linearity range of 0.005–5.5 mM and a lower detection limit of 0.21 mM was reported by Shao et al. (2017). The high sensitivity of the biosensor can be attributed to its porous structure with the formation of efficient detection channels, which lead to an improvement in electron transport and the interaction between PANI and analyte (AA) (Shao et al., 2017). The HNTs were used to make a sensor for the detection and quantification of tumor biomarkers. In a study by Li et al. (2017b), based on a core–shell structure, particles of palladium (Pd) were grafted onto the surface of HNTs coated with polypyrrole (PPy) (HNT@PPy-Pd). This hybrid nanocomposite was fabricated as an analytical signal label for the quantitative detection of the tumor marker, prostate specific antigen (PSA), a prostate cancer biomarker. The sensor exhibited acceptable reproducibility, selectivity, and stability, with a lower detection limit of 0.03 pg/mL. The development scheme of the PSA biosensor (Li et al., 2017b) is shown in Fig. 8.

In conclusion, despite the limited number of studies conducted on the use of HNTs in the construction of biosensors, characteristics such as high biocompatibility, easy availability, selective modification for specific targeting, and dimensions in the order of nanometers offer potential applications for nanotubes in this area.

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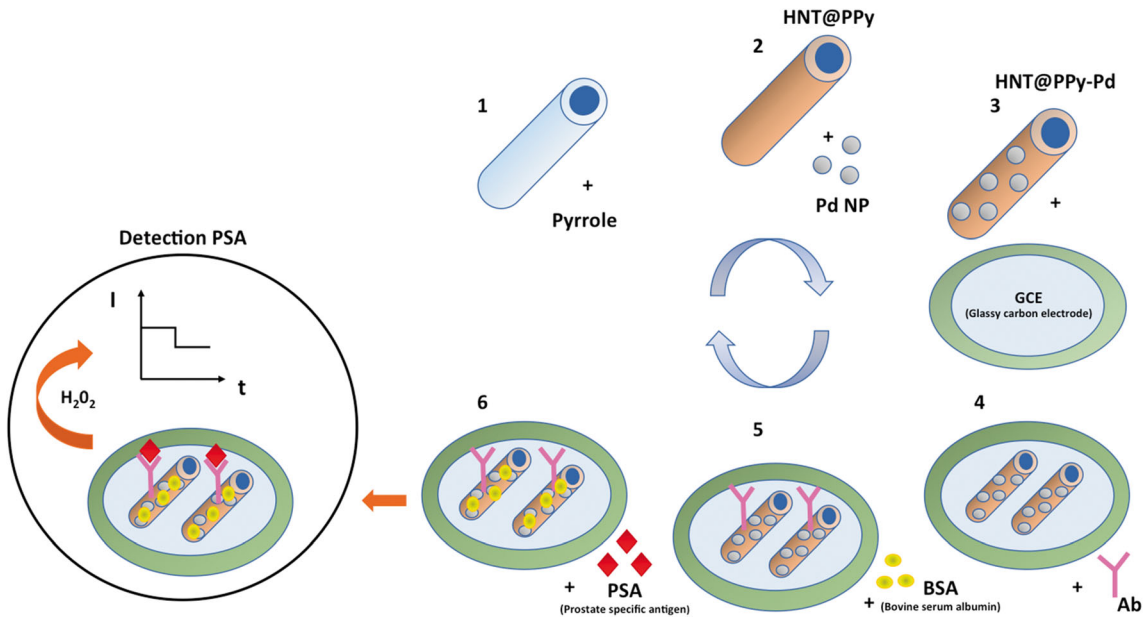


Fig. 8. Development phases of the PSA biosensor

FUTURE DIRECTIONS AND CONCLUSIONS

The main properties of HNTs have been described, including cytocompatibility, considerable surface area, tubular structure, and nanometric dimensions, and are useful for those working on the construction of nanodevices. In addition, the small cost, the wide availability in nature, and the ability to improve the mechanical characteristics have led to growing interest in materials based on HNTs as a multifunctional biomaterial in biomedical applications. The hollow lumen is an essential feature for the encapsulation of bioactive molecules and for their gradual and controlled release near or within a desired cell; and finally, the high aspect ratio of the tubular nanosystem favors penetration through the cell membrane. In addition, HNTs are formed by laminated layers of aluminosilicate which create a structure with a positively charged hollow lumen and a negatively charged external surface, all of which lead to good dispersibility in alcohol, water, and polar polymers. The inner lumen with a diameter of 10–40 nm allows for 10–20 wt.% drug and protein load, with a prolonged release of the therapeutic agent up to 10–20 h, which can be extended to 100 h with the termination of the nanotube or with its inclusion in a polymer matrix. In addition, the opposite distribution of charge between the external and internal surfaces allows selective immobilization of the therapeutic agents. To all this is added the functional versatility of HNTs, which includes modification to silanes and LbL self-assembly, as well as functionalization with targeted fractions (for safer and more efficient drug release), including folic acid, biotin, and materials which respond to specific enzymes (e.g. end caps of dextrins). This allows for selective cellular internalization and triggered drug release for anticancer applications.

Several biologically active molecules have been loaded into HNTs. Such pharmaceutical applications include the entrapment of anticancer, anti-inflammatory, antibiotic, analgesic, antihistamine, corticosteroid, and cardiovascular drugs. In addition, HNTs have shown themselves to be promising nano-platforms for the immobilization of enzymes and nucleic acids in clinical diagnostics and therapy. Entrapment in HNTs preserves the activity of enzymes, providing protection from degradation by proteases. The HNTs have resulted in an improvement in the use of enzymes in colon, cervical, and breast cancer. In addition, HNTs have emerged as non-viral gene-delivery agents (less toxic than carbon nanotubes and cationic-nucleic acid polymer complexes) for DNA, siRNA, and oligonucleotides, showing high transfection efficiency and gene silencing which confirm promising application of HNTs as vectors in gene therapy.

The development of scaffolds from HNTs and polymers is an exciting direction in the design of emerging materials in biomedicine. HNTs represent a promising material in the structuring of polymer matrices. The grafting of just 3–6% by weight of HNTs leads to significant changes in the physicochemical characteristics of the resulting materials. The known properties of HNTs such as solubility, hydrophilicity/hydrophobicity, and mechanical strength are manipulated for their application in various fields such as biosensing, wound healing, and hydrogel fillers in tissue engineering. The application of HNTs in regenerative medicine and tissue engineering has been very successful, particularly in bone and dental repair, and in hair surface engineering. The realization of biomimetic architectures has led to improved proliferation, adhesion, and differentiation of

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stem cells. The grafting of HNTs allows improvement in the mechanical characteristics of the scaffolds, in addition to the absorption of water. In addition, the encapsulation of bioactive molecules in the lumen of HNTs allows the development of functional matrices or membranes which release antibiotics to inhibit infections and promote faster wound healing. Likewise, the grafting of antibiotic-loaded HNTs at the bone cement level has shown important potential in clinical applications in orthopedic replacement surgery. The use of HNTs in the immobilization of enzymes associated with the bone biomineralization process is a further step forward in bone repair. Halloysite nanotubes have extraordinary potential, therefore, in the production of multifunctional, high-performance HNT-polymer nanocomposites, showing improved mechanical, thermal, and flame-retardant properties, as well as a prolonged drug-release capacity and good hemocompatibility/cytocompatibility. Other possible biomedical applications of HNT-polymer nanocomposites, such as biosensors and cancer diagnosis (bioimaging), remain to be developed, however. In addition, the toxicology of HNTs should be investigated thoroughly; a weakness is the absence of comprehensive studies evaluating the pharmacokinetics, toxicity, and safety of HNTs, in humans, rather than in vitro or in vivo in animals. The toxicological evaluation of HNTs is in its infancy and must be further investigated; because HNTs are natural nanomaterials, their properties vary according to their origin, with different toxicological profiles. Future research will elucidate fully the long-term effect of HNTs (uncontaminated and modified) on human health. An important aspect for an advance in the application of HNTs in biomedicine is related to the safety of this material which usually has different impurities with which different toxic effects can be associated. It will be essential to introduce new technologies that enable greater purity of the materials. Furthermore, hybrid clay-drug nanosystems are very effective in vitro, though some of them fail in vivo due to complications with real physiological conditions. The most valid formulations seem to be topical cosmetic formulations. The focus on topical application is related to the fact that this nanomaterial is not readily biodegradable in the blood, limiting its use in intravenous formulations, due to the risk of thrombosis.

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Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

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