Nanoformulations based on collagenases loaded into halloysite/Veegum® clay minerals for potential pharmaceutical applications

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Abstract: The design and development of nanomaterials capable of penetrate cancer cells is fundamental when anticancer therapy is involved. The use of collagenase (Col) is useful since this enzyme can degrade collagen, mainly present in the tumor extracellular matrix. However, its use is often limited since collagenase suffers from inactivation and short half-life. Use of recombinant ultrapure collagenase or carrier systems for their delivery are among the strategies adopted to increase the enzyme stability. Herein, based on the more stability showed by recombinant enzymes and the possibility to use them in anticancer therapy, we propose a novel strategy to further increase their stability by using halloysite nanotubes (HNTs) as carrier. ColG and ColH were supramolecularly loaded onto HNTs and used as fillers for Veegum gels. The systems could be used for potential local administration of collagenases for solid tumor treatment. All techniques adopted for characterization showed that halloysite interacts with collagenases in different ways depending with the Col considered. Furthermore, the hydrogels showed a very slow release of the collagenases within 24 h. Finally, biological assays were performed by studying the digestion of a type-I collagen matrix highlighting that once released the Col still possessed some activity. Thus we developed carrier systems that could further increase the high recombinant collagenases stability, preventing their inactivation in future *in vivo* applications for potential local tumor treatment.

Keywords: halloysite; collagenases; hydrogels, Veegum®, sustained release.

1. Introduction

Nanomaterials have become a powerful weapon in the fight against cancer because of their passive accumulation within solid tumors (the EPR effect). However, despite their small size (1-100 nm), nanomaterials cannot efficiently reach tumor sites through the EPR effect,[1] since the presence of tumor extracellular matrix (ECM) which is denser than healthy tissues and has higher collagen content, hinders the nanomaterial penetration into cancer cells, limiting the therapeutic efficacy. Over the years, different strategies have been adopted to overcome these limitations, and some of them foresee the possibility of using collagenases (Col), specific enzymes that, once activated by natural cofactors such as Ca^{2+} and Zn^{2+}, can degrade the collagen fibers. In addition, several studies have shown that *in situ* administration of collagenase breaks down ECM collagen barriers, increasing the drug penetration in ECM accumulated tissue.[2, 3] However, these enzymes, by extractive procedures, suffer from a short half-life and inactivation, due to proteolytic contaminants, which limit their *in vivo* applications. To avoid these, different strategies were adopted. One foresees the possibility to obtain more stable collagenases, another the use of carrier systems which protect the enzyme, preserving their activity for longer times and, ensuring accumulation in the tumor tissue.[4] Few examples deal with the synthesis of collagenases with increased stability. In this context, Ghersi *et al.* have developed a protocol to synthetize ultrapure collagenases by DNA recombination techniques,[5] which showed an improved enzyme stability compared to the ones extracted by *Clostridium hystoliticum*.[6] Indeed, both recombinant Class I (ColG) and Class II (ColH) collagenases showed improved stability in solution for at least 18 days which depends to their intrinsic activity on collagen.[7, 8]

As far as the delivery of Col is concerned, Schroeder *et al.* reported the synthesis of a collagozome, namely collagenase loaded into liposomes for the treatment of pancreatic tumor. The authors demonstrated that the delivery of the enzyme with liposomes enhances the therapeutic efficacy by protecting collagenase from deactivation, modulating its release profile inside the tumor, favoring drug uptake.[9] Acetalated dextran were used to deliver collagenases through tumor tissue. By means of this system, the authors achieved high Col loading efficiency, preservation of the enzymatic activity and controlled release in a mildly acidic microenvironment.[10] Pegylated gold nanoparticles were decorated with Col allowing their accumulation in tumor tissue 35-fold higher than pristine ones.[11] Clay minerals, because of their high biocompatibility, high availability, and low cost, represent emerging materials for application in biomedical field.[12, 13] In the last years, there has been an increasing interest in the utilization of clay minerals not only as excipients in pharmacological formulations but also as active ingredients for the delivery of different drugs.[14-16] In this context, halloysite, a clay mineral with a typical hollow tubular structure, holds several advantages for biomedical applications.[17]

Halloysite, with a general formula of $Al_2Si_2O_5(OH)_4 \cdot n(H_2O)$, is an aluminosilicate clay mainly found in the form of nanotubes (due to this, it is also known as halloysite nanotubes, HNTs), constituted by 10-15 rolled sheets where siloxane groups are located at the external surface, whereas aluminol groups are present in the lumen. Due to this peculiar structure and chemical composition, the tubes undergo ionization in aqueous media, conferring on the clay a negatively charged external surface and a positively charged inner one in a wide pH range.[18]

The different surface charges and the presence of an empty lumen mean that several molecules can be loaded onto HNTs by exploiting supramolecular interactions such as electrostatic attraction interactions, hydrophobic effects, or hydrogen bonding interactions.[19-22] By this approach, several anticancer drugs, antioxidant molecules, and so on have been loaded, and the biological effects of the resulting nanomaterials have been evaluated by *in vitro* and *in vivo* assays.[23-27] For example, by exploiting the different charged surface of HNTs, proteins or enzymes were selectively immobilized at the external surface or loaded into the lumen by working in pH ranges above or below their isoelectric points.[28, 29]

The use of HNTs as filler for hydrogel matrices, led to the synthesis of nanocomposite gels with improved rheological properties for the *in-situ* administration of active species. With this approach, it was possible to obtain slow-release systems for therapeutic applications.[30-35]

Recently, the effects of HNTs as filler in inorganic hydrogels formed by clay minerals, were studied.[36, 37] Clay minerals belonging to the smectite groups are indeed, able to form hydrogels in aqueous media due to the formation of delaminated dispersions by the self-assembling of their nanodisks via face-edge aggregation.[38] The obtained hydrogels are biocompatible, stable, and thixotropic, and due to these features, they are widely used in pharmacological applications. HNTs can efficiently interact with the smectite nanodisks by electrostatic attraction interactions helping the gel formation and improving the rheological properties.[36, 37] Among the different smectite clay minerals employed for the above purposes, the magnesium silicate clay known as Veegum® HS (purified bentonite) is one of the most commercialized clays of pharmacological grade.[39]

Herein, based on the more stability showed by recombinant enzymes and the possibility to use them in cell therapy and tissue engineering applications, we propose a novel strategy to further increase their stability by using halloysite as carrier. To do this, ColG and ColH were supramolecularly loaded onto HNTs obtaining the nanomaterials HNTs/ColG and HNTs/ColH. To the best of our knowledge this is the first example of recombinant collagenases [5] delivered by an inorganic carrier used as filler for smectite hydrogel.

We developed a novel carrier system that could further increase the high recombinant collagenases stability, by their loading in HNTs, preventing their inactivation in future *in vivo* applications for

potential local tumor treatment. The interaction between halloysite and both collagenases was assessed by Resonance Light Scattering (RLS) measurements and the obtained nanomaterials were thoroughly characterized by thermogravimetric analysis (TGA), FT-IR spectroscopy, DLS and ζ-potential measurements and their morphologies were investigated by transmission electron microscopy (TEM) coupled with energy-dispersive X-ray (EDX). Then, we explored the possibility of obtaining Veegum® hydrogels in the presence of HNTs/ColG and HNTs/ColH (Figure 1). The mechanical properties of the hydrogels obtained were examined by rheology measurements. Furthermore, the kinetic release from both HNTs based nanomaterials and nanocomposite hydrogels was investigated in conditions mimicking the physiological ones.

The non-loss of activity of the collagenases loaded on the halloysite was also evaluated and quantified in comparison to the same collagenases in free form.

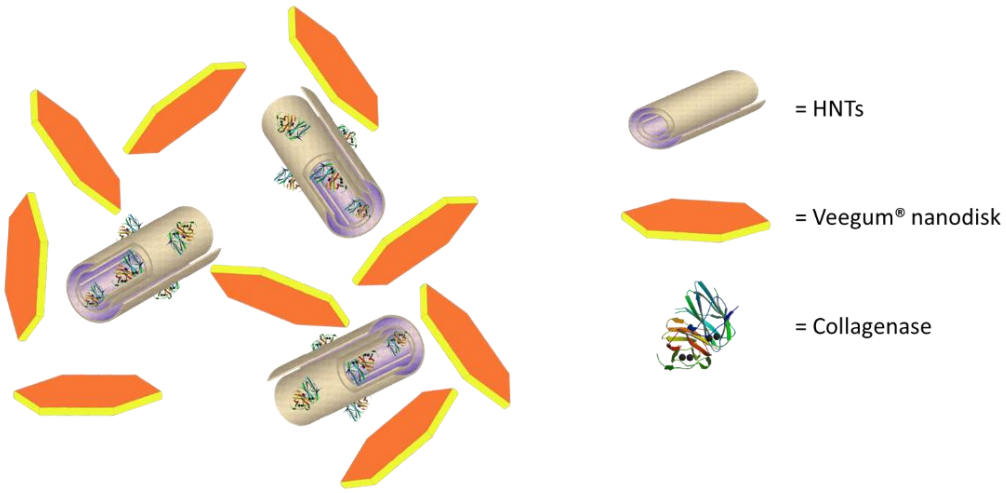


Figure 1. Schematic representation of the designed system for the potential delivery of collagenase in tumor tissues.

2. Materials and Methods

The ultrapure recombinant collagenases ColG (145 kDa) and ColH (149 kDa) were provided by Abiel s. r. l. (Palermo, Italy); unlike other commercial collagenases, of extractive origin which contain various contaminants with proteolytic activity, the collagenases produced by ABIEL srl are recombinant enzymes of class I (ColG) and class II (ColH) of *Clostridium hystoliticum*, which have been optimized to be produced in *Escherichia coli*, in the strain BL21 Ai, with a purity >99% and totally free of contaminants with enzymatic activity.[40]

Halloysite nanotubes used in this study were obtained from Merck and used as received.

FT-IR spectra (KBr) were acquired with an Agilent Technologies Cary 630 FT-IR spectrometer.

The size analysis, ζ-potential and polydispersity index of the samples were determined using a Malvern Zetasizer Nano ZS instrument, fitted with a 532 nm laser at a fixed scattering angle of 173°. RLS measurements were performed at 25 °C on a spectrofluorophotometer (Jasco FP-777 W) equipped with a system for temperature control. A synchronous scanning mode was used, and monochromators of emission and excitation were preset to identical wavelengths. The RLS spectrum was recorded from 200 to 750 nm with both the excitation and emission slit widths set at 1.5 nm.
Thermogravimetric analysis was carried out on Q5000 apparatus (TA Instruments).
Transmission electron microscopy (TEM) was performed by means of a FEI Titan G2 60–300 ultra-high-resolution transmission electron microscope (FEI, Lausanne, Switzerland) coupled with analytical electron microscopy (AEM) performed with a SUPER X silicon drift windowless energy dispersive X-ray spectroscopy (XEDS) detector. AEM spectra were saved in mode STEM (scanning transmission electron microscopy) with a HAADF (high angle annular dark field) detector.
Rheological measurements were performed by a controlled rate viscometer (Thermo Scientific HAAKE, RotoVisco 1) equipped with a plate/plate combination (Ø 20mm serrated PP20S sensor system). Temperature was maintained constant during the whole experiment at 25 °C (± 0.5 °C) by means of a temperature controller. Rheological properties of hydrogel samples were measured within the shear rate range of 70-600 s^{-1}. The measurement interval was chosen to simulate typical stresses that may suffer these systems when used: 70-200 s^{-1}(skin spreading), 100-200 s^{-1} (manual mixing) and 400-2000 s^{-1} (container removal). Rheological characterization was performed after 48 h (swelling time). Data were collected and processed by the HAAKE RheoWin software. Six replicates were obtained for each sample. Rheological characterization included flow curves and apparent viscosity taken at 270 s^{-1}.

2.1 Loading of Collagenases onto HNTs

To a dispersion of pristine HNTs in ethanol (100 mg, 5 mL), a solution of Collagenase G or Collagenase H in H_2O (10 mg mL^{-1}, 1 mL) was added. The obtained dispersion was sonicated (5 min, ultrasound power of 200 W, 25 °C), evacuated for 3 cycles and finally it was left under stirring for 18 h at room temperature. After this time, the dispersion was centrifuged and the obtained powder was washed with ethanol, until the unreacted collagenases were removed and then lyophilized.

2.2 Gel preparation

Nanocomposite hydrogels were prepared by weighing into a screw-capped sample vial (diameter 2.5 cm) the Veegum® (0.5 g), HNTs based nanomaterials (0.025 g) and solvent (ca. 5 g). The mixture was first dispersed for 5 minutes with ultrasound irradiation and left at room temperature until a gel was obtained.

2.3 Thixotropic and Sonotropic Behaviour

The gel phases obtained were subjected to two different external stimuli. The mechanical stimulus was involved by stirring the gel phase with a stirring bar of 8 mm of length and 3 mm of height at 1000 rpm for 5 min. The sonotropic behaviour of the gel phases was tested by irradiating in an ultrasound water bath for 5 min with a power of 200 W and a frequency of 45 kHz. Thereafter, the materials were stored at room temperature overnight. When the samples were stable to the tube-inversion test, the gels were defined as thixotropic or sonotropic.

2.4 Kinetic release from HNTs based nanomaterials and Veegum® hydrogels

30 mg of HNTs/ColG or HNTs/ColH were dispersed in 2 mL of deionized water. The dispersion was putted into a polypropylene one-way syringe equipped with a polytetrafluoroethylene (PTFE) frit. After predetermined time the aqueous phase was removed under vacuum and analysed by UV–vis measurements at λ of 275 nm, to determine the collagenases concentration by the Lambert-Beer law. To ensure sink conditions, 2 mL of fresh deionized water were added to remaining powder to replace the collected ones.

As far as is regarding collagenase release from hydrogels it was done as follows: nanocomposite hydrogels obtained in water at 10 wt % of Veegum® and 5 wt% of HNTs/Col were prepared, as discussed above, in a total volume of 5 mL. 5 mL of the gelation solvent were casted on gel matrix. The release kinetic was carried out at 37 °C. At fixed intervals of time, 250 μL of supernatant solution were taken out to be analyzed by zymography (see *infra*), and simultaneously refilled with other 250 μL of the same solvent pre-warmed at 37 °C.

Total amounts of collagenases released (F_t) in both cases were calculated as follows:

$$F_t = V_m C_t + \sum_{i=0}^{t-1} V_a C_i \qquad \text{(Eq. 1)}$$

where V_m and C_t are the volume and the concentration of the collagenase at time *t*. V_a is the volume of the sample withdrawn and C_i is the collagenase concentration at time *i* ($i < t$).

2.5 Zymography

The ColG and ColH samples, obtained by the HNTs/ColG/Veegum® and HNTs/ColH/Veegum® release assay, were loaded into an electrophoretic zymography gel containing 7.5% Acrylamide and 0.6 mg/mL gelatin. Briefly, after the electrophoretic run, the gel was washed two times with a buffer containing 2.5% Triton X-100 and NaN_3 0.02%, then incubated for 24 h at 37 °C in a development buffer Tris-HCl buffer (50 mmol/L; pH 7.4), containing 1.5% Triton X-100 and 0.02% Na Azide plus 2 mmol/L $CaCl_2$. The gel was stained with Coomassie Blue R 250 0.8% in Met-OH - CH_3COOH -

H_2O in a proportion 5:1:5; after 1 h staining the zymography gel was destained in the same solution of staining deprived of the colorant.[41] The zymographic approach permits to obtain of clear and sharp bands of the digested substrate against a dark background of the undegraded substrate. The bands in the gel are quantified by ImageJ 64 program in comparison to know amount in weight of pure ColG or ColH.

2.6 Collagen gel digestion assay

3D rat collagen type-I gels (BD Biosciences, San Jose, CA, USA) were prepared in Hank's buffer (4 mg/mL) adding of Trypan Blue (0% v/v) and neutralized with NH_4HCO_3 to allow collagen polymerization. Gels of 1 mL of collagen were prepared in each well of a 24-well plate and incubated at 37 °C to facilitate the polymerization. Following, 10 µL of HNTs/ColG or HNTs/ColH were spotted in each gel and incubated at 37 °C for 6 hours. Gels incubated with 10 µL of Phosphate Buffered Saline (PBS) were used as a negative control; while gels treated with 0.45 mg/mL of collagenases G or H (free/ColG or Free/ColH) were used as positive control.

2.7 Quantitative collagen enzymatic activity

Enzymatic activity of ColG or ColH released from HNTs samples was quantitatively evaluated by the ninhydrin-based assay Collagenase Substrate Kit (Sigma-Aldrich, Milan) according to manufacturer's instructions and in the presence of carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH synthetic peptide, with some modification [42, 43]. The assay was performed in two steps: in the first step, 100 µL of each sample were incubated with 500 µL of the substrate (2.4 mg/mL in TES Buffer, pH 7.0 at 37 °C for 5 min. During the second step, 150 µL of each sample were incubated with the Ninhydrin solution and Tin(II) chloride at 100 °C for 15 min.
Collagenase activities were evaluated by spectrophotometric analysis at 565 nm: under these conditions, Ninhydrin interacts with the free amino groups of the synthetic substrate hydrolyzed by collagenase developing the colorimetric reaction directly proportional to the enzymatic activity. The enzymatic activity, expressed as U/mg, was calculated by using a calibration curve.

3. Results and Discussion

The synthesis of the nanocomposite hydrogels for the potential delivery of collagenases into tumor tissue preventing their inactivation was accomplished in two different steps. Firstly, both ColG and ColH were loaded onto HNTs, developing nanomaterials that were subsequently used as fillers for Veegum® hydrogels.

3.1 Loading of ColG and ColH into HNTs

To obtain information about the interaction of collagenase G and collagenase H with halloysite we performed preliminary studies by means of resonance light scattering (RLS) measurements. This is a kind of elastic scattering technique which is due to the fluctuations of solution refraction index. From a chemical point of view it is important since this technique allows the detection of the presence of aggregates in solution and thus it can be efficiently used to study aggregation processes between organic molecules and HNTs surfaces.[31, 44] The experiment was performed by titrating a HNTs dispersion (0.1 mg mL^{-1}) with an increasing concentration of collagenase G or collagenase H in a concentration range of 0–0.04 mg mL^{-1} at 25 °C. Figure 2a shows the trend of RLS intensity at 500 nm as a function of collagenase concentration. As is it possible to observe the RLS intensity of HNTs increases with increasing of collagenase concentration in both cases investigated. These findings indicate the occurrence of interactions between HNTs and collagenases and therefore the presence of nanoparticles with greater dimensions. The larger intensity of RLS scattering in the presence of ColH in comparison to that of ColG could indicate a stronger interaction between HNTs with ColH rather than with ColG.

The loading of ColG and ColH into pristine HNTs was carried out by a consolidate procedure adopted for this kind of interaction. To maximize the enzyme loading onto HNTs, an ethanolic HNTs suspension was evacuated in the presence of concentrated enzyme aqueous solution (10 mg mL^{-1}). Ethanol was chosen because of in this solvent Col is not soluble and thus, the interaction with HNTs is favored. After loading, the HNTs/ColG and HNTs/ColH nanomaterials were washed several times with ethanol to remove free enzyme molecules. Enzyme loading of both nanomaterials was estimated by TGA analysis by using the rule of mixtures, which is commonly employed for the determination of the composition of halloysite based systems.[45] Being HNTs negatively charged on the external surface and positively charged in the lumen, the occurrence of electrostatic attraction interaction between the HNTs surfaces and the carboxylate or ammonium portion of Col were envisaged. The loading efficiencies, expressed as the percent amount of enzyme in the final nanomaterial, were ca. 2.1 wt% and ca. 4.5 wt% for HNTs/ColG and HNTs/ColH, respectively. The greater loading obtained in the case of ColH in comparison to that of ColG led us to hypothesize that ColH could interact with both HNTs surfaces.

TGA allowed us also to investigate the thermal behavior of the obtained HNTs based nanomaterials. Compared to the pristine HNTs, both nanomaterials are stable up to ca. 350 °C, when a first degradation occurs, indicating the presence of organic matter in the nanomaterials (Figure 2b). Afterwards, the typical degradation of HNTs at ca. 550 °C, due to the expulsion of interlayer water molecules, is observed after which stable values up to 800 °C were observed.

The obtained nanomaterials were also characterized by FT-IR spectroscopy. The FT-IR spectra of both nanomaterials (Figure 2c) show the typical vibration stretching bands of HNTs[46] with some additional vibration bands. In particular, in the range 1650 – 1550 cm^{-1} are clear observable the bands related to the amide I (1648 cm^{-1}), amide II (1563 cm^{-1}) and C–N (1470 cm^{-1}) stretching[47] (Figure S.1) beside the typical bending band of –OH groups of interstitial water of pristine HNTs (1630 cm^{-1}) overlapped by the Col signals, which further confirm the presence of collagenases in the HNTs/ColG and HNTs/ColH nanomaterials.

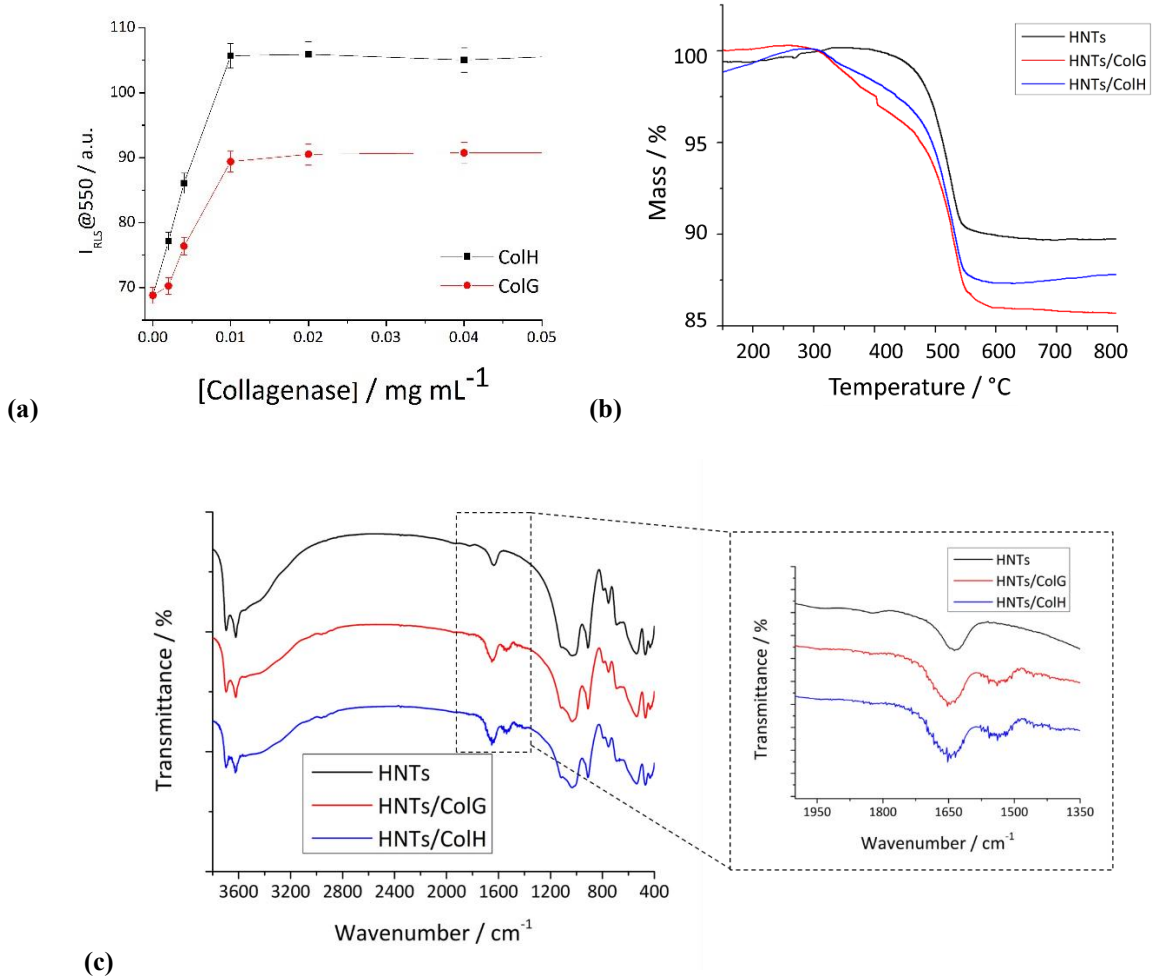


Figure 2. (a) Trend of RLS intensity as a function of collagenase concentration (ranging from 0 to 0.04 mg mL^{-1}). Reported are the mean ± standard deviation values; (b) thermogravimetric curves of pristine HNTs, HNTs/ColG and HNTs/ColH nanomaterials; (c) FT-IR spectra of HNTs/ColG and HNTs/ColH nanomaterials.

The diffusion dynamics of HNTs/ColG and HNTs/ColH nanomaterials in aqueous dispersion were characterized by dynamic light scattering (DLS) experiments. DLS measurements, as already reported for other systems,[48] allow the determination of the structural characteristics of the nanomaterials by monitoring their mobility in water and by measuring the average translational

diffusion coefficient. This coefficient considers the shape, dimension, and hydration of the diffusing particles and also the existence of aggregation phenomena.

A single translational diffusion mode was observed in the case of HNTs/ColG dispersion with Z-average sizes (i.e., the intensity weighted mean diameters), calculated by applying the Stokes–Einstein equation, of ca. 530 ± 30 nm (similar to that of pristine HNTs, 570 ± 40 nm) and a polydispersity index (PDI) of 0.653. These findings indicated that the ColG interacts preferentially with the HNTs lumen. On the contrary, the HNTs/ColH nanomaterial showed an increase in the dimensions in comparison to pristine HNTs; it was indeed observed a Z-average size value of ca. 1200 ± 100 nm with a PDI of 0.874. In this case it was hypothesized that ColH interacts with both HNTs surfaces in agreement with the highest enzyme loading obtained for HNTs/ColH nanomaterial in comparison to the HNTs/ColG one.

ζ-potential measurements highlighted a slight decrease in the ζ-potential values of both nanomaterials in comparison to that of pristine HNTs (-16 mV), being them -18.5 and -19.2 mV, for HNTs/ColG and HNTs/ColH, respectively, because of the interaction with the enzymes, these indeed, being rich of carboxylate moieties render the ζ-potential values of the final nanomaterials slightly more negative than that of pristine HNTs.

TEM and HAADF/STEM images of both nanomaterials show that after the loading of collagenases the tubular morphology of HNTs was preserved. However, while the HNTs/ColG nanomaterial appears as well dispersed tubes, the morphology of HNTs/ColH nanomaterial is different. Indeed, in this case the tubes seem to be agglomerated because of ColH interaction with the external surface. The latter finding agrees to TGA and DLS measurements. Energy-dispersive X-ray spectroscopy (EDS) elemental mapping showed that in the HNTs/ColG nanomaterial, the ColG molecules were present mainly in the HNTs lumen; whereas, the HNTs/ColH nanomaterial showed the presence of the enzyme on the overall surface of the tubes, as highlighted by the distribution of N atoms (Figure 3B and 4B) in both nanomaterials. Moreover, close observation of the tubes (Figure 3C and 4C) showed the presence of N atoms colocalized in the lumen as far as is regarding the HNTs/ColG nanomaterial (Figure 3C) as proven by EDS elemental mapping performed along the HNTs section (selected area in green); whereas them are localized both on the external surface and in the lumen in the case of HNTs/ColH nanomaterials (Figure 4C). All these findings confirm the presence of Col onto HNTs surfaces beings that pristine HNTs does not show trace of N atoms (Figure S.2a).

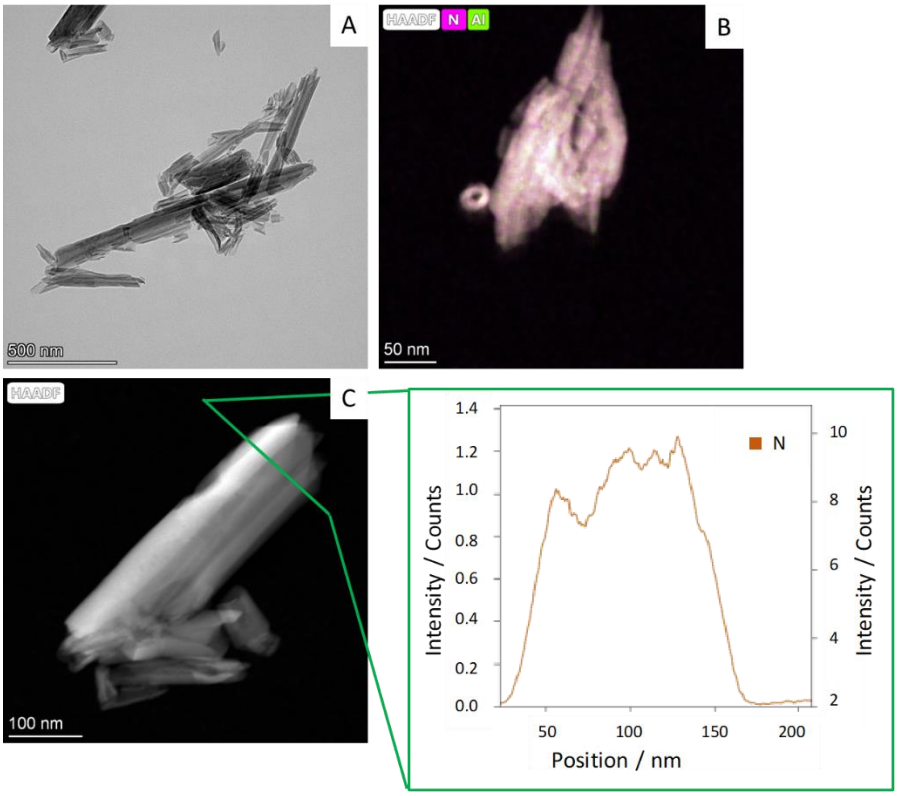


Figure 3. (A) TEM images of HNTs/ColG nanomaterial. (B) EDX elemental mapping image from the HAADF/STEM image. (C) EDS elemental mapping of N atoms along the selected area of the HAADF-STEM image of the HNTs/ColG nanomaterial.

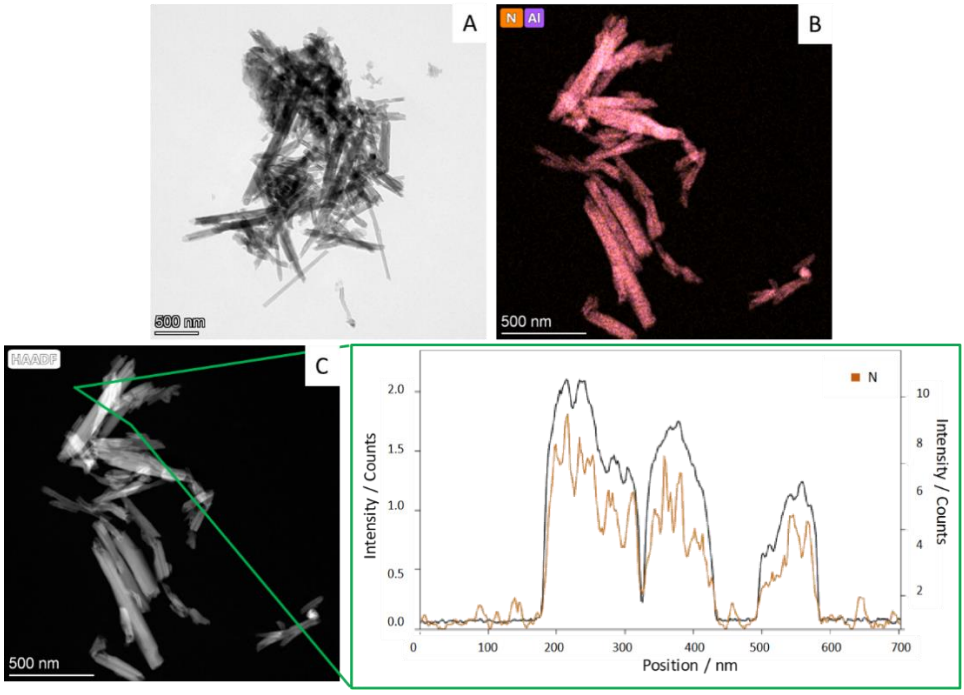


Figure 4. (A) TEM images of HNTs/ColH nanomaterial. (B) EDX elemental mapping image from the HAADF/STEM image. (C) EDS elemental mapping of N atoms along the selected area of the HAADF-STEM image of the HNTs/ColH nanomaterial.

3.2 Kinetic release of ColG and ColH from HNTs/ColG and HNTs/ColH

As proof of concept of the feasibility of the obtained nanomaterials for the delivery of collagenases into tumor site, we investigated the kinetic release of both ColG and ColH from the HNTs/ColG and HNTs/ColH in water (pH 7.0). The release profile of both collagenases is shown in Figure 5. As it is possible to observe both systems show a faster release of collagenases in the first 5 min where about 50 wt% of the total amount of both ColG and ColH loaded is released, followed by a plateau after 24 h. To better understand the release mode of the molecules from the HNTs carrier, the kinetic data were analyzed by the first-order and double-exponential (DEM) models. The mathematical analysis showed that the kinetic release of ColG from HNTs/ColG nanomaterial follow a first order model (k = 0.015 ± 0.002 min^{-1}, R^2 = 0.9953) indicating a diffusion of ColG from the HNTs lumen over the time, whereas the ColH release from the HNTs/ColH nanomaterial is better described by a DEM model (R^2 = 0.9999). Since DEM model discriminate between two distinguishable species, in the latter case, we observed a fast desorption from the HNTs surface (k_1 = 0.392 ± 0.006 min^{-1}) followed by a slower release from the HNTs lumen (k_2 = 0.021 ± 0.001min^{-1}) in agreement with that described above.

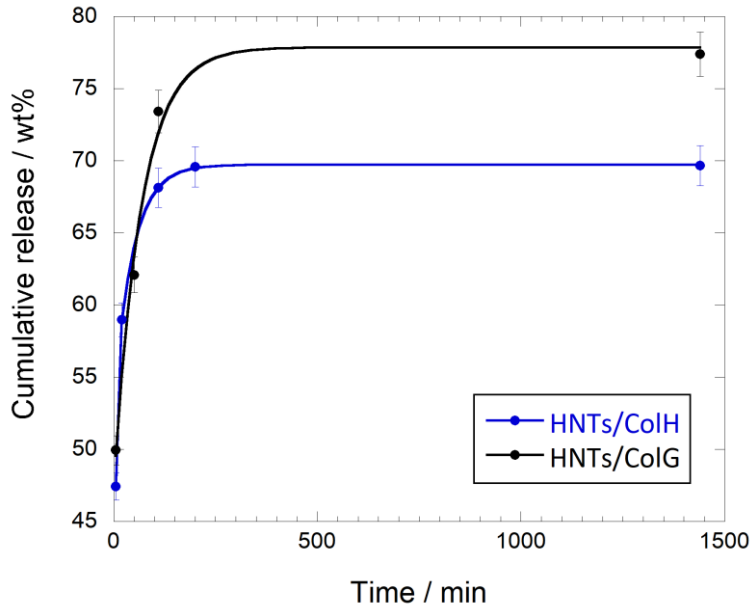


Figure 5. Kinetic release of ColG and ColH from HNTs/ColG and HNTs/ColH, respectively, in water. Reported are the mean ± standard deviation values.

3.3 Studies of gel properties

As known Veegum® is a natural clay mineral belonging to the smectite group which possesses a pharmacological grade. As other smectite clays it can form stable hydrogels in aqueous media due to the formation of delaminated dispersions by the self-assembling of its nanodisk via face-edge

aggregation [38]. It is well documented that the addition of HNTs filler to clay hydrogels lead to an improvement of the mechanical properties of the resulting gel [36]. In addition, the use of hydrogels for pharmacological applications, is commonly employed to slow down the release of active species in biological fluids.[37] Therefore, herein we studied first the influence of HNTs, HNTs/Col H and G fillers on the mechanical properties of the Veegum® hydrogel and then we evaluated the kinetic release of collagenases from the hydrogel matrix.

3.3.1 Rheological properties

Veegum® based hydrogel demonstrated self-repair after disruption, confirming their thixotropic nature, and they resulted stable to ultrasound irradiation, these properties were also confirmed by rheological measurements. Flow curves (shear rate vs. shear stress) of hydrogels are plotted in Figure 6a. All the hydrogels behave as non-Newtonian, pseudoplastic materials, in which is characteristic the progressive decline in shear stress slope as shear rate increased. Also, all samples showed a hysteresis area, correlated to thixotropy, i.e., application of shear rate determined a reversible rupture of the internal network, the recovery of which gradually went on once the applied stress was removed or reduced. From the flow curves, it was possible to obtain the apparent viscosities (at 270 s^{-1}) (Figure 6a), being HNTs/ColG gel the one with the highest viscosity value and the highest profile of the curve. Therefore, the formulation with HNTs/ColG leads to improved rheological properties. Similar results were obtained in the case of pristine HNTs. These findings further confirm the selective interaction of ColG with HNTs lumen. The highest viscosity obtained in this case, is in agreement with literature findings about the introduction of HNTs filler in smectite based hydrogels.[37] On the other hand, and in agreement with the agglomeration of tubes observed in TEM and HAADF/STEM images, flow curve of gels prepared with HNTs/ColH nanomaterial greatly decrease the profile, corresponding to a reduction in apparent viscosity associated to the interaction of ColH with the external surface of the nanotubes.

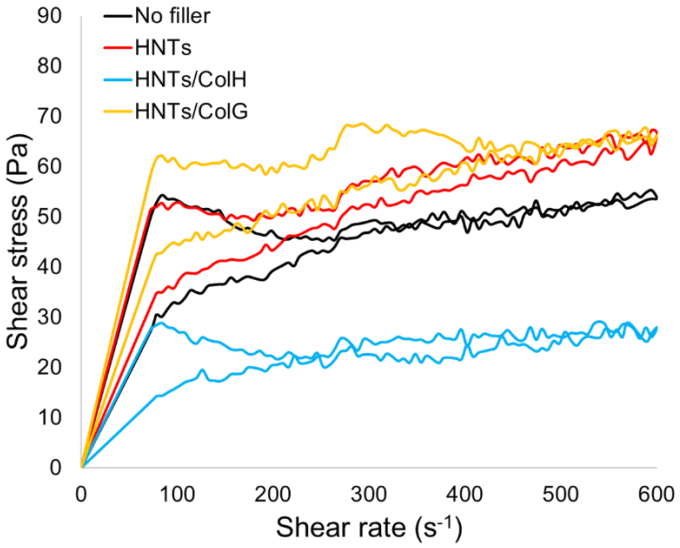
3.3.2 Kinetic release

The kinetic release of both ColG and ColH from the HNTs/ColG/Veegum® and HNTs/ColH/Veegum® nanocomposite hydrogels, was investigated in water (pH 7.0) at 37 °C to verify if incorporation of HNTs into the gel matrix ensures a time-controlled Col release process. The amount of collagenases release was evaluated by measuring the enzymatic activity by a gelatin zymography as report in M&M (Figure 6b).

The trends of cumulative Col release from the hybrid gels as a function of time are shown in Figure 6c. As it is possible to observe, in both cases, the hydrogels retain almost the totality of collagenase loaded for at least 24 h indicating that can act as reservoir for the sustained release of collagenases

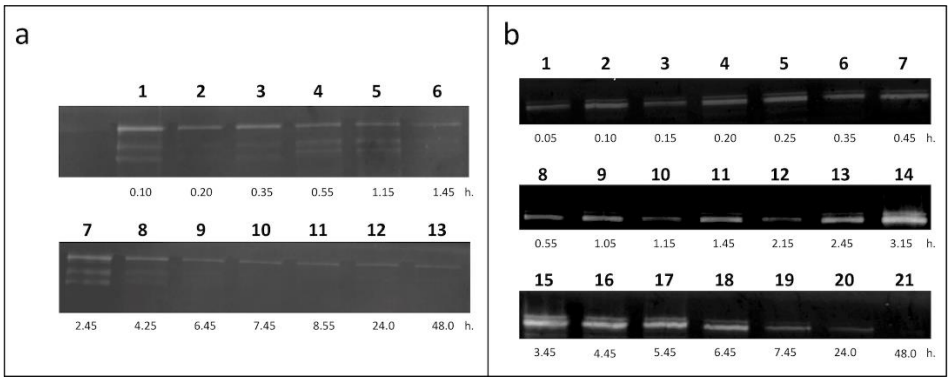
within the time. Although the release rate is low in both cases, ColH is released in slightly greater amount in comparison to ColG in agreement with its loading at both HNTs surfaces. The mathematical fitting of the experimental kinetic data indicates that the release of both ColG and ColH from HNTs/ColG/Veegum® and HNTs/ColH/Veegum® is ruled by a simple diffusion through gel matrix being the data better described by a first order model (M_∞= 0.043±0.001 wt%, k= 0.04±0.003 min^{-1} and M_∞= 0.072±0.003 wt%, k= 0.013±0.001 min^{-1} for ColG and ColH, respectively).

The slower release observed in the case of hybrid hydrogels in comparison to the Col release from HNTs could be explained taking into account the existence of some favorable electrostatic attraction interactions between the Col released from the filler and the Veegum® nanoparticles which further slow down the enzyme release.

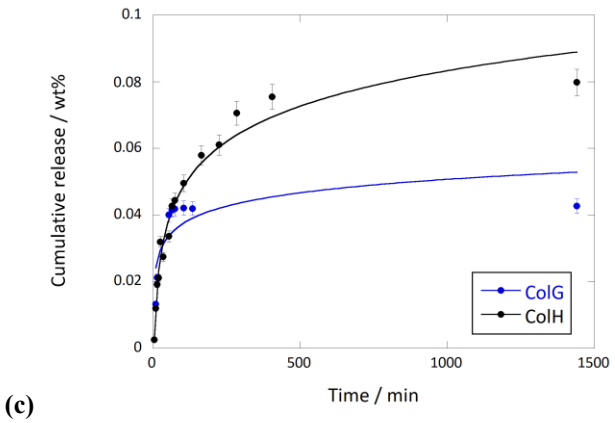


Filler into Veegum®	η (Pa s)
/	0.17 ± 0.03
HNTs	0.20 ± 0.02
HNTs/ColH	0.09 ± 0.03
HNTs/ColG	0.250 ± 0.003

(a)



(b)



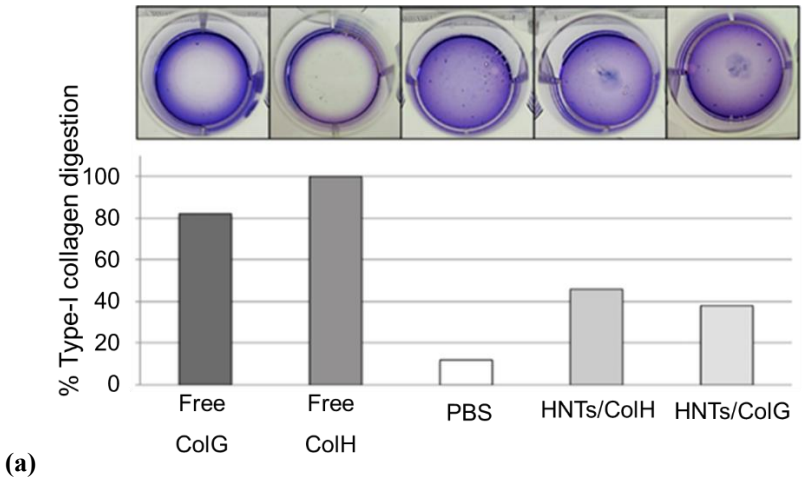
(c)

Figure 6. (a) Flow curves of hydrogels (mean values; n = 6); (b) Gelatin zymography of enzymatic activity of collagenases released from (i) HNTs/ColG/Veegum® and (ii) HNTs/ColH/Veegum® nanocomposite hydrogels at different times; (c) Kinetic release of ColG and ColH from HNTs/ColG/Veegum® and HNTs/ColH/ Veegum® hydrogels, respectively, in water, at 37 °C. Reported are the mean ± standard deviation values.

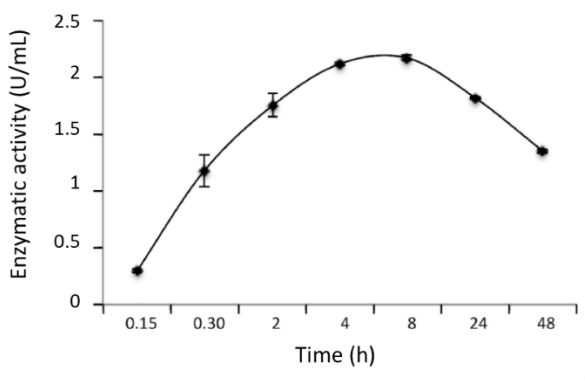
3.4 Biological Properties

Finally, we performed preliminary investigation to verify if the collagenases retain their enzymatic activity once loaded on HNTs. In particular, the biological activity was evaluated by an *in vitro* assay studying the digestion of a type-I collagen matrix. The effect of the free formulation collagenases (Free/ColG and Free/ColH) was compared with a control sample treated with an equivalent volume of phosphate-buffered saline (PBS) and with the collagenases loaded on HNTs (HNTs/ColG and HNTs/ColH) after a 6 h treatment, in comparison with alcian blue stained collagen gel at time 0 (Figure 7a).

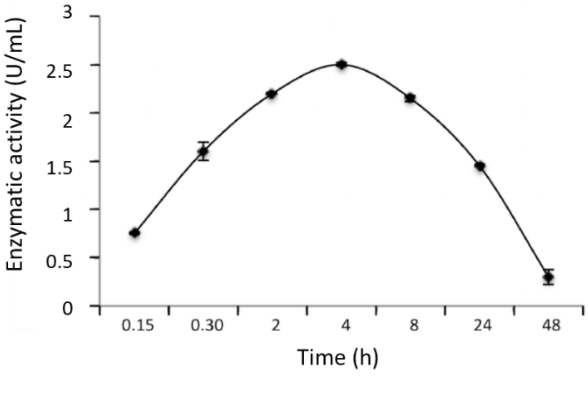
As shown in Figure 7a, after 4 h of treatment the free formulation collagenases have a digestive effect massive on the type-I collagen gel, in particular, Free/ColH has digested the matrix 100% while for Free/ColG about 82%; this different digestion efficiency is attributable to the fact that ColG, as reported in the literature [7, 8] works exclusively on three-dimensional structures of the substrate, while ColH recognizes linear sequences of collagen. As regards the enzymes loaded on HNTs, only a partial digestion by HNTs/ColG and HNTs/ColH was observed, attributable to a protective action by the nanomaterials that contain them and to the release kinetics of these. As shown in Figure 7b-c, the release of collagenases from the nanotubes is delayed over time by the analysis of the enzyme activity (Pz) found in the solution. This latter result is encouraging since it shown that by delivering Col by means of HNTs, it is possible to obtain a nanosystem for the controlled and sustained release of the enzyme, preventing its inactivation but at the same time retaining the enzymatic activity.



(a)



(b)



(c)

Figure 7. (a) Type-I collagen digestion by ColG and ColH in free formulation (Free/ColG and Free/ColH) respect enzymes loaded into HNTs (HNTs/ColG and HNTs/ColH) compared to non-treated gel; (b) evaluation in collagenases activity as Pz (U/mL) of released ColG (a) and ColH (b) from HNTs in time (reported in hours). Reported are the mean ± standard deviation values.

5. Conclusions

In summary, we report the use of halloysite nanotubes as carriers for two kind of collagenase, collagenase G (ColH) and collagenase H (ColH).

The synthesis of the HNTs/Col nanomaterials was verified by several techniques that shown that the collagenases interact in different way with halloysite. Firstly, the affinity of Col for HNTs surfaces was investigated by RLS measurements. Afterwards, it was demonstrated that ColG interacts mainly with HNT lumen, as proved by DLS measurements and morphological investigations. In the latter case indeed, by acquiring the elemental mapping of N atoms by EDX along an HNT section, it was possible to verify the presence of the organic molecules in the lumen proximity. Conversely, ColH was found at both surfaces as testified by the DLS value greater than that of pristine HNTs and by the morphological investigations. Kinetic release showed that both Col are sustained released from the carrier reaching ca. 50 wt% of the total collagenase release after 24 h.

To obtain systems for *in situ* administration of the nanomaterials, as example for treatment of solid tumors, HNTs/Col were used as Veegum® fillers. The experimental data showed that the presence of the inorganic filler makes the formation of stable hydrogels with the better rheological performances showed by HNTs/ColG, further confirming the presence of the enzyme into HNTs lumen. Kinetic release from the nanocomposite hydrogels showed a very slow release over the time. Finally biological assays on the digestion capacity of the nanomaterials towards collagen I showed that the Col released from the tubes still possessed some enzymatic activity.

Thus, by delivering enzymes by means of HNTs, offers the possibility to combine the clay properties with the ones of enzyme and to obtain nanosystems for the delivery of the enzyme into cancer tumor matrix by degrading the ECM, preventing its early inactivation, modulating the release and retaining the enzymatic activity. Therefore, the advantages provide by the obtained systems can be resumed as:

- the HNTs carriers are able to convey the collagenases in an inactive form, which in any case retains their enzymatic properties when released at the level of the site of action (high Ca^{++} concentration), the tumor extracellular matrix;
- the enzymes release is modulated over time by the presence both HNTs and Veegum® gels;
- the used enzymes are ultrapure ones, and this could suggest their specific effect on the collagenous component of the tumor extracellular matrix, allowing for solid tumors the decompaction and thus favoring the penetration with high efficiency of regulatory drugs or biomolecules into the cells tumors;
- the combination of all properties conferred by the systems could finally led to the elimination of the tumor mass, increasing the EPR effect, inducing the reduction of the hydrostatic pressure at the level of the tumor vascular network.
- Finally, being ultrapure enzymes, the presence of unwanted side effects is eliminated, because of these are generally attributed to lytic activities induced by proteolytic components contaminating the collagenases used.

Future works will be devoted to study the co-delivery of chemotherapeutic agents and the enzymes for the treatment of different diseases. Since HNTs is not biodegradable, it is possible to hypothesize a future use of the developed systems for local administration for the treatment of primary tumors.

Author Contributions:

M. Massaro: methodology; investigation, writing—original draft preparation, writing—review and editing.

G. Ghersi: conceptualization, investigation, investigation, writing—original draft preparation, writing—review and editing., funding acquisition.

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S. Campora: methodology; investigation, writing—original draft preparation.

S. Rigogliuso: methodology; investigation, writing—original draft preparation.

R. Sànchez-Espejo: methodology; investigation, writing—original draft preparation.

C. Viseras-Iborra: methodology; investigation, writing—original draft preparation, writing—review and editing.

S. Riela: G. Ghersi: conceptualization, investigation, investigation, writing—original draft preparation, writing—review and editing., funding acquisition.

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References

[1] S. Campora, G. Ghersi, Recent developments and applications of smart nanoparticles in biomedicine, Nanotechnology Reviews, 11 (2022) 2595-2631.
[2] M.R. Villegas, A. Baeza, M. Vallet-Regí, Hybrid Collagenase Nanocapsules for Enhanced Nanocarrier Penetration in Tumoral Tissues, ACS Applied Materials and Interfaces, 7 (2015) 24075-24081.
[3] J. Li, C. Xie, J. Huang, Y. Jiang, Q. Miao, K. Pu, Semiconducting Polymer Nanoenzymes with Photothermic Activity for Enhanced Cancer Therapy, Angewandte Chemie - International Edition, 57 (2018) 3995-3998.
[4] L. Zhou, Q. Liang, Y. Li, Y. Cao, J. Li, J. Yang, J. Liu, J. Bi, Y. Liu, Collagenase-I decorated co-delivery micelles potentiate extracellular matrix degradation and hepatic stellate cell targeting for liver fibrosis therapy, Acta Biomaterialia, 152 (2022) 235-254.
[5] PCTWO2011/073925A9, in.
[6] M. Salamone, A. Cuttitta, F. Bertuzzi, C. Ricordi, G. Ghersi, G. Seidita, Biochemical comparison between Clostridium hystoliticum collagenases G and H obtained by DNA recombinant and extractive procedures, in: Chemical Engineering Transactions, 2012, pp. 259-264.
[7] O. Matsushita, K. Yoshihara, S. Katayama, J. Minami, A. Okabe, Purification and characterization of Clostridium perfringens 120-kilodalton collagenase and nucleotide sequence of the corresponding gene, Journal of bacteriology, 176 (1994) 149-156.
[8] M. Salamone, S. Saladino, M. Pampalone, S. Campora, G. Ghersi, Tissue Dissociation and Primary Cells Isolation Using Recombinant Collagenases Class I and II, Chemical Engineering Transactions, 38 (2014) 247-252.
[9] A. Zinger, L. Koren, O. Adir, M. Poley, M. Alyan, Z. Yaari, N. Noor, N. Krinsky, A. Simon, H. Gibori, M. Krayem, Y. Mumblat, S. Kasten, S. Ofir, E. Fridman, N. Milman, M.M. Lübtow, L. Liba, J. Shklover, J. Shainsky-

Roitman, Y. Binenbaum, D. Hershkovitz, Z. Gil, T. Dvir, R. Luxenhofer, R. Satchi-Fainaro, A. Schroeder, Collagenase Nanoparticles Enhance the Penetration of Drugs into Pancreatic Tumors, ACS Nano, 13 (2019) 11008-11021.
[10] J. Wang, Q. Wu, Y. Wang, L. Xiang, J. Feng, Z. Zhou, Q. Fu, L. Zhang, Collagenase-loaded pH-sensitive nanocarriers efficiently remodeled tumor stroma matrixes and improved the enrichment of nanomedicines, Nanoscale, 13 (2021) 9402-9414.
[11] S. Murty, T. Gilliland, P. Qiao, T. Tabtieng, E. Higbee, A.A. Zaki, E. Puré, A. Tsourkas, Nanoparticles Functionalized with Collagenase Exhibit Improved Tumor Accumulation in a Murine Xenograft Model, Particle & Particle Systems Characterization, 31 (2014) 1307-1312.
[12] D. Peixoto, I. Pereira, M. Pereira-Silva, F. Veiga, M.R. Hamblin, Y. Lvov, M. Liu, A.C. Paiva-Santos, Emerging role of nanoclays in cancer research, diagnosis, and therapy, Coordination Chemistry Reviews, 440 (2021) 213956.
[13] M. Massaro, R. Ciani, G. Cinà, C.G. Colletti, F. Leone, S. Riela, Antimicrobial Nanomaterials Based on Halloysite Clay Mineral: Research Advances and Outlook, Antibiotics, 11 (2022).
[14] J. Dong, Z. Cheng, S. Tan, Q. Zhu, Clay nanoparticles as pharmaceutical carriers in drug delivery systems, Expert Opinion on Drug Delivery, 18 (2021) 695-714.
[15] C. Viseras, P. Cerezo, R. Sanchez, I. Salcedo, C. Aguzzi, Current challenges in clay minerals for drug delivery, Applied Clay Science, 48 (2010) 291-295.
[16] M. Notarbartolo, M. Massaro, R. de Melo Barbosa, C. Emili, L.F. Liotta, P. Poma, F.M. Raymo, R. Sànchez-Espejo, R. Vago, C. Viseras-Iborra, S. Riela, Exploring the cellular uptake of hectorite clay mineral and its drug carrier capabilities, Colloids and Surfaces B: Biointerfaces, 220 (2022).
[17] D. Ganapathy, R. Shanmugam, S. Pitchiah, P. Murugan, A. Chinnathambi, S.A. Alharbi, K. Durairaj, A.K. Sundramoorthy, Potential Applications of Halloysite Nanotubes as Drug Carriers: A Review, Journal of Nanomaterials, 2022 (2022) 1068536.
[18] M. Massaro, F. Armetta, G. Cavallaro, D.F. Chillura Martino, M. Gruttadauria, G. Lazzara, S. Riela, M. d'Ischia, Effect of halloysite nanotubes filler on polydopamine properties, Journal of Colloid and Interface Science, 555 (2019) 394-402.
[19] O. Prinz Setter, L. Dahan, H. Abu Hamad, E. Segal, Acid-etched Halloysite nanotubes as superior carriers for ciprofloxacin, Applied Clay Science, 228 (2022) 106629.
[20] D. Sid, M. Baitiche, R. Bourzami, R. Merir, F. Djerboua, A. Gil, M. Boutahala, Experimental and theoretical studies of the interaction of ketoprofen in halloysite nanotubes, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 627 (2021) 127136.
[21] M. Saleh, N. Prajapati, A. Karan, N. Rahman, A. Stavitskaya, M. DeCoster, Y. Lvov, Halloysite Nanotube Vehicles for Drug Delivery Through a Model Blood–Brain Barrier, Clays and Clay Minerals, 69 (2021) 603-611.
[22] G. Cavallaro, S. Milioto, S. Konnova, G. Fakhrullina, F. Akhatova, G. Lazzara, R. Fakhrullin, Y. Lvov, Halloysite/Keratin Nanocomposite for Human Hair Photoprotection Coating, ACS Applied Materials & Interfaces, 12 (2020) 24348-24362.
[23] A.A. Oun, A.Y. Bae, G.H. Shin, M.-K. Park, J.T. Kim, Comparative study of oregano essential oil encapsulated in halloysite nanotubes and diatomaceous earth as antimicrobial and antioxidant composites, Applied Clay Science, 224 (2022) 106522.
[24] A. Stavitskaya, G. Fakhrullina, L. Nigamatzyanova, E. Sitmukhanova, E. Khusnetdenova, R. Fakhrullin, V. Vinokurov, Biodistribution of Quantum Dots-Labelled Halloysite Nanotubes: A Caenorhabditis elegans In Vivo Study, Materials, 14 (2021) 5469.
[25] M. Massaro, S. Pieraccini, S. Guernelli, M.L. Dindo, S. Francati, L.F. Liotta, G.C. Colletti, S. Masiero, S. Riela, Photostability assessment of natural pyrethrins using halloysite nanotube carrier system, Applied Clay Science, 230 (2022) 106719.
[26] M. Massaro, M. Notarbartolo, F.M. Raymo, G. Cavallaro, G. Lazzara, M.M.A. Mazza, C. Viseras-Iborra, S. Riela, Supramolecular Association of Halochromic Switches and Halloysite Nanotubes in Fluorescent Nanoprobes for Tumor Detection, ACS Applied Nano Materials, 5 (2022) 13729-13736.
[27] Y. Long, Y. Feng, Y. He, B. Luo, M. Liu, Hyaluronic Acid Modified Halloysite Nanotubes Decorated with ZIF-8 Nanoparticles as Dual Chemo- and Photothermal Anticancer Agents, ACS Applied Nano Materials, 5 (2022) 5813-5825.

[28] J. Tully, R. Yendluri, Y. Lvov, Halloysite Clay Nanotubes for Enzyme Immobilization, Biomacromolecules, 17 (2016) 615-621.
[29] M. Massaro, G. Cavallaro, C.G. Colletti, G. D'Azzo, S. Guernelli, G. Lazzara, S. Pieraccini, S. Riela, Halloysite nanotubes for efficient loading, stabilization and controlled release of insulin, Journal of Colloid and Interface Science, 524 (2018) 156-164.
[30] L.W. Wong, P. Pasbakhsh, W.T. Cheng, C.B.S. Goh, J.B.L. Tan, One-pot synthesis of injectable self-healing thermoresponsive halloysite nanotube-reinforced nanocomposite hydrogels for tissue engineering, Applied Clay Science, 232 (2023) 106812.
[31] H. Ghasemizadeh, M. Pourmadadi, F. Yazdian, H. Rashedi, M. Navaei-Nigjeh, A. Rahdar, A.M. Díez-Pascual, Novel carboxymethyl cellulose-halloysite-polyethylene glycol nanocomposite for improved 5-FU delivery, International Journal of Biological Macromolecules, 232 (2023) 123437.
[32] S. Zolfagharian, P. Zahedi, M. Shafiee Ardestani, A. Khatibi, S. Jafarkhani, Sodium alginate/xanthan-based nanocomposite hydrogels containing 5-fluorouracil: Characterization and cancer cell death studies in presence of halloysite nanotube, Journal of Industrial and Engineering Chemistry, 120 (2023) 374-386.
[33] C. Rizzo, R. Arrigo, F. D'Anna, F. Di Blasi, N.T. Dintcheva, G. Lazzara, F. Parisi, S. Riela, G. Spinelli, M. Massaro, Hybrid supramolecular gels of Fmoc-F/halloysite nanotubes: Systems for sustained release of camptothecin, Journal of Materials Chemistry B, 5 (2017) 3217-3229.
[34] X. Lin, Y. Feng, Y. He, S. Ding, M. Liu, Engineering design of asymmetric halloysite/chitosan/collagen sponge with hydrophobic coating for high-performance hemostasis dressing, International Journal of Biological Macromolecules, 237 (2023) 124148.
[35] Y. Feng, Y. He, X. Lin, M. Xie, M. Liu, Y. Lvov, Assembly of Clay Nanotubes on Cotton Fibers Mediated by Biopolymer for Robust and High-Performance Hemostatic Dressing, Advanced Healthcare Materials, 12 (2023) 2202265.
[36] M. Massaro, A. Borrego-Sánchez, R. Sánchez-Espejo, C. Viseras Iborra, G. Cavallaro, F. García-Villén, S. Guernelli, G. Lazzara, D. Miele, C.I. Sainz-Díaz, G. Sandri, S. Riela, Ciprofloxacin carrier systems based on hectorite/halloysite hybrid hydrogels for potential wound healing applications, Applied Clay Science, 215 (2021) 106310.
[37] M. Massaro, G. Buscemi, L. Arista, G. Biddeci, G. Cavallaro, F. D'Anna, F. Di Blasi, A. Ferrante, G. Lazzara, C. Rizzo, G. Spinelli, T. Ullrich, S. Riela, Multifunctional Carrier Based on Halloysite/Laponite Hybrid Hydrogel for Kartogenin Delivery, ACS Medicinal Chemistry Letters, 10 (2019) 419-424.
[38] P. Shi, Y.-H. Kim, M. Mousa, R.R. Sanchez, R.O.C. Oreffo, J.I. Dawson, Self-Assembling Nanoclay Diffusion Gels for Bioactive Osteogenic Microenvironments, Advanced Healthcare Materials, 7 (2018) 1800331.
[39] K. Asare-Addo, A.-M. Totea, A. Nokhodchi, Drug release from magnesium aluminium silicate-polyethylene oxide (PEO) nanocomposite matrices: An investigation using the USP III apparatus, European Journal of Pharmaceutical Sciences, 153 (2020) 105474.
[40] L. Volpe, M. Salamone, A. Giardina, G. Ghersi, Optimization of a Biotechnological Process for Production and Purification of Two Recombinant Proteins: Col G and Col H, Chemical Engineering Transactions, 49 (2016) 61-66.
[41] Z. Ren, J. Chen, R.A. Khalil, Zymography as a Research Tool in the Study of Matrix Metalloproteinase Inhibitors, in: J. Wilkesman, L. Kurz (Eds.) Zymography: Methods and Protocols, Springer New York, New York, NY, 2017, pp. 79-102.
[42] W. Grassmann, A. Nordwig, [Quantitative colorimetric test for collagenase], Hoppe-Seyler's Zeitschrift fur physiologische Chemie, 322 (1960) 267-272.
[43] M. Salamone, S. Rigogliuso, A. Nicosia, M. Tagliavia, S. Campora, P. Cinà, C. Bruno, G. Ghersi, Neural Crest-Derived Chondrocytes Isolation for Tissue Engineering in Regenerative Medicine, Cells, 9 (2020) 962.
[44] M. Massaro, E. Licandro, S. Cauteruccio, G. Lazzara, L.F. Liotta, M. Notarbartolo, F.M. Raymo, R. Sánchez-Espejo, C. Viseras-Iborra, S. Riela, Nanocarrier based on halloysite and fluorescent probe for intracellular delivery of peptide nucleic acids, J. Colloid Interface Sci., 620 (2022) 221-233.
[45] S. Riela, A. Barattucci, D. Barreca, S. Campagna, G. Cavallaro, G. Lazzara, M. Massaro, G. Pizzolanti, T.M.G. Salerno, P. Bonaccorsi, F. Puntoriero, Boosting the properties of a fluorescent dye by encapsulation into halloysite nanotubes, Dyes and Pigments, 187 (2021) 109094.

[46] M. Massaro, M. Casiello, L. D'Accolti, G. Lazzara, A. Nacci, G. Nicotra, R. Noto, A. Pettignano, C. Spinella, S. Riela, One-pot synthesis of ZnO nanoparticles supported on halloysite nanotubes for catalytic applications, Applied Clay Science, 189 (2020) 105527.
[47] A. Kishen, S. Shrestha, A. Shrestha, C. Cheng, C. Goh, Characterizing the collagen stabilizing effect of crosslinked chitosan nanoparticles against collagenase degradation, Dental Materials, 32 (2016) 968-977.
[48] S. Riela, A. Borrego-Sánchez, S. Cauteruccio, R. de Melo Barbosa, M. Massaro, C.I. Sainz-Díaz, R. Sánchez-Espejo, C. Viseras-Iborra, E. Licandro, Exploiting the interaction between halloysite and charged PNAs for their controlled release, Journal of materials chemistry. B, 11 (2023) 6685-6696.