Halloysite Clay Nanotubes for Resveratrol Delivery to Cancer Cells

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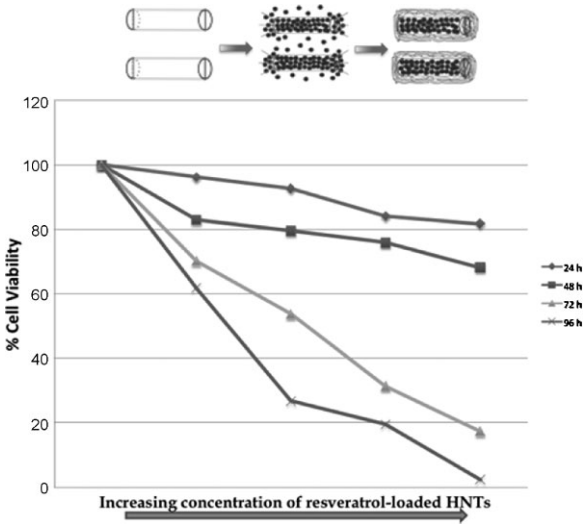
Halloysite is natural aluminosilicate clay with hollow tubular structure which allows loading with low soluble drugs using their saturated solutions in organic solvents. Resveratrol, a polyphenol known for having antioxidant and antineoplastic properties, is loaded inside these clay nanotubes lumens. Release time of 48 h is demonstrated. Spectroscopic and ζ-potential measurements are used to study the drug loading/release and for monitoring the nanotube layer-by-layer (LbL) coating with polyelectrolytes for further release control. Resveratrol-loaded clay nanotubes are added to breast cell cultures for toxicity tests. Halloysite functionalization with LbL polyelectrolyte multilayers remarkably decrease nanotube self-toxicity. MTT measurements performed with a neoplastic cell lines model system (MCF-7) as function of the resveratrol-loaded nanotubes concentration and incubation time indicate that drug-loaded halloysite strongly increase of cytotoxicity leading to cell apoptosis.



1. Introduction

Resveratrol, a polyphenolic compound that can be found in red grapes, peanuts, as well as a variety of other plant sources, is one of the natural compounds that have been intensively investigated in recent years for its health-beneficial properties, and for potential applications in the fields of pharmaceutics, nutraceuticals and functional foods.[1] Resveratrol is well known for its high antioxidant activity and is considered a key component for the health benefits of red wine. Chemically, resveratrol exist in two geometrical isomers, *trans*-resveratrol and *cis*-resveratrol, however, *trans*-resveratrol is the natural form of isomer, and the more stable and the most active form.[2]

Clinical studies have demonstrated several health-promoting activities of *trans*-resveratrol, such as anti-oxidizing[3] and anti-artherosclerotic effects, inhibition of platelet aggregation[4] and polymorphonuclear leukocyte function,[5–6] beneficial effects on the cardiovascular system, reducing lipid peroxidation, improving vasodilatation and lowering blood pressure[7] and chemo-protective advantages against cancer proliferation.[8]

Most recently, resveratrol has been shown to improve general health and survival of mice on a high-caloric diet, pointing to new approaches for treating obesity-related disorders and diseases of aging.[9]

Interestingly, resveratrol has also estrogenic activity, being able to function as an ERα agonistic ligand.[10–12] Recent reports have analyzed the anti proliferative activity

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of resveratrol in human cancer cell lines of diverse origin. Others have reported that resveratrol inhibited cell viability and proliferation in the human breast cancer cell lines MCF-7 and MDA-MB-231, regardless of their estrogen receptor (ER) status.[13–15] However, whereas the ERα positive MCF-7 cells exhibited apoptotic cell death, the ERα negative MDA-MB-231 did not,[14] suggesting that apoptosis induction by resveratrol in human breast cancer cells could be ERα mediated. Vergara et al. have demonstrated that resveratrol induces down-regulation of the protein cyclin D1. The phosphorylation level of the protein kinase B (Akt) and glycogen synthase-3b (GSK-3b), two kinases potentially involved in the regulation of cyclin D1, was decreased in resveratrol treated samples. The phosphorylation of the mammalian target of rapamycin (mTOR), a downstream target of Akt, was also modulated by resveratrol.

Finally, resveratrol treatment resulted in the down-regulation of phosphorylated-extracellular signal-regulated kinase (pERK), and the combination of phosphatidylinositol 3-kinase (PI3K) and ERK inhibitors with resveratrol further enhanced resveratrol effects on cell proliferation.[16]

However, the physicochemical properties of resveratrol, particularly its high reactivity and low solubility in aqueous and lipid phases, have been limiting factors for its bioavailability and efficacies of the desired health-beneficial effects. Resveratrol is highly soluble in alcohols, whereas only soluble in trace amounts in aqueous or lipid phase.[17] In addition, resveratrol is a very reactive molecule, very susceptible to react with dissolved oxygen, producing different degradation products, as well as very easily degraded by sunlight.[18] In a biological system, resveratrol is rapidly and extensively metabolized, probably due to its low water solubility, which reduces the dissolution-rate limited cell absorption,[19] thus reducing its oral bioavailability. Therefore, exploitation of resveratrol as pharmaceutical compound is only feasible when encapsulated in a delivery system, which is capable of stabilizing and protecting it from degradation, while preserving its biological activities and enhancing its bioavailability. Encapsulation is one of such potential systems. However, until now, only few studies have addressed the suitability of delivering encapsulated resveratrol to the site of action, and the main research focus has been its biological activity, especially in synergy with the consumption of other diet/beverage components[20] or chemotherapeutic agents.

More specifically, encapsulation of resveratrol in delivery systems of nanometric size has been found to contribute even more significantly to the improvement of its cell uptake. For examples, nanometric carriers for pharmaceutical applications were designed using lipospheres to efficiently transport resveratrol into the cardiovascular system,[21] while biodegradable polymeric nanoparticles[22] and liposomes were developed to enhance resveratrol chemo-preventive efficiency.[20] More recently, polymeric micelles have been used to enhance the ability of resveratrol to protect cells from oxidative stress and apoptosis,[23] and solid lipid nanoparticles, to increase the uptake of resveratrol in keratinocytes cells.[24] In addition, cyclodextrins have been used as a carrier molecule to increase both bioavailability and stability of resveratrol through the formation of inclusion complexes.[25]

In recent years, nanoscale formulations allowed for essential progress in pharmaceuticals. Nanoparticle drug formulation combined with the design of nano-containers for controlled release, targeting, and making availability of low soluble drugs are in development.[26–27] Bio-responsive for biomimetic materials used to create drug delivery systems typically includes synthetic polymers and natural materials such as lipids, polysaccharides and proteins.

One such nanosized delivery system is naturally available clay nanotubes called halloysite (Figure 1). Halloysite was found to be a viable and inexpensive nanoscale container for the encapsulation of biologically active molecules such as biocides and drugs, first demonstrated by Price, Lvov et al.[28–29] The neighboring alumina and silica layers, and their water hydration, create a packing disorder causing them to curve and roll up, forming multilayer tubes.[30] The use of halloysite offers significant advantages over other nanotubes. Because it occurs in nature, production is neither tedious nor hazardous. It is inexpensive in comparison to other nanotubes in use (carbon nanotubes and inorganic nanotubes made of tungsten, titanium, etc.).[31]

Halloysite is tubular aluminosilicate clay and in our previous work[32] we characterized it as tubular

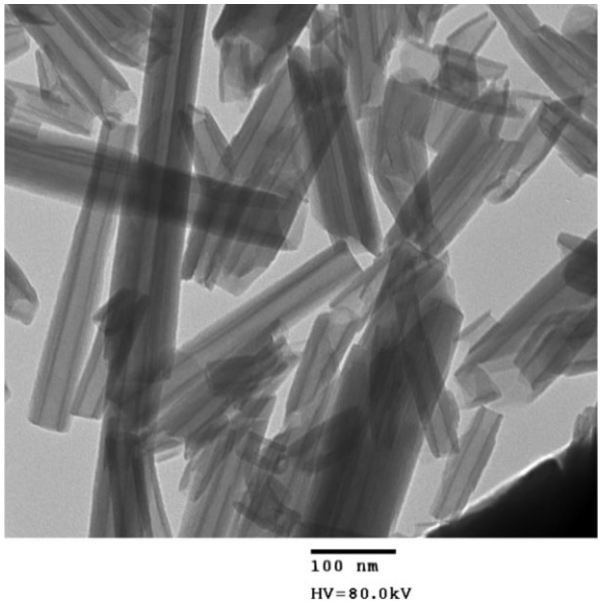


Figure 1. TEM of halloysite clay nanotubes.

nanoparticles of ca. 50 nm external diameter, 15 nm lumen diameter, and of 500–1 500 nm length. In wide range of pH, it has negative electrical ζ potential of ca. −50 mV, which allows halloysite good dispersibility and colloidal stability in water. We also demonstrated that halloysite nanotubes are nontoxic up to concentrations of 75 μg/mL, whereas parallel laser confocal visualization of cell uptake of fluorescently labeled halloysite demonstrated its location within the cells in the nuclear vicinity. The biocompatibility of halloysite and the internalization by cells are the main prerequisites for safe usage of them in the delivery of biologically active substances in medical and household products.

In this work, we have used halloysite nanotubes to encapsulate resveratrol. We have studied their release profile and demonstrated the efficacy of the polyphenolic compound on MCF-7 (breast cancer cell line) as apoptotic agent. Finally we have also shown how to slow down the release rate using the layer-by-layer technique by closing nanotubes' lumens.

2. Experimental Section

2.1. Chemicals

Chemicals suppliers were as follows: polyethyleneimine (PEI) was purchased from Sigma, USA; poly(sodium 4-styrene-sulfonate) (PSS) was purchased from Sigma, USA; poly(allylamine hydrochloride) (PAH) from Sigma, USA; protamine salt, grad III (PRM) from Sigma, USA; dextran sulfate sodium salt from Leuconostoc spp. (DXS) Sigma, USA; fetal bovine serum (FBS) from Sigma, USA; penicillinstreptomycin solution (Sigma), sodium pyruvate (Sigma), DMEM medium (Sigma); thiazolyl blue tetrazolium bromide >97.5% (TLC) from Sigma, USA; phosphate-buffered saline, Dulbecco A (PBS) from Oxoid; Resveratrol (R5010 Sigma). Purified dehydrated halloysite was obtained from Applied Minerals, Inc and used without further treatment.

2.2. Loading Procedure

In order to encapsulate resveratrol inside halloysite nanotubes, we have mixed 2 mg of halloysite, as dry powder, with few mL of saturated solution of resveratrol in 70% ethanol. The Eppendorf containing the mixture was soaked and transferred in a vacuum jar. By using a vacuum pump this mixture was then evacuated. Suspension was kept under vacuum for 30 up to 90 min, and then was cycled back to atmospheric pressure. This process was repeated five times in order to increase loading efficiency. All processing has taken 30 minutes excluding vacuum drying time. At the end resveratrol-loaded nanotubes were centrifuged and washed with distilled water.

2.3. Entrapment Efficiency

After loading procedure, the halloysite suspension was centrifuged at 6.500 rpm for 5 min and was washed three times with distilled water and the supernatant was used for measurement. Halloysite nanotubes without resveratrol were used as blank.

Resveratrol entrapment efficiency (EE) was determined with the following equation:

$$EE\,(\%) = C_{\mathrm{HNTs}} = (V_{\mathrm{i}}C_{\mathrm{i}} - V_{\mathrm{s}}C_{\mathrm{s}})/V_{\mathrm{HNTs}} * 100$$

Where C_{HNTs} is the concentration of resveratrol associated to halloysite, C_{i} and C_{s} represent drug feeding concentration and drug concentration in supernatant after incubation, respectively; V_{i}, V_{s}, V_{HNTs} refer to the volume of drug feeding solutions, the volume of supernatant, and the volume of halloysite, respectively. The concentration in supernatant is derived from UV-Vis absorption referring to a standard curve.

2.4. Release Kinetics

The release kinetics was evaluated in phosphate buffer at physiological pH. Halloysite and resveratrol mixture was constantly stirred in order to increase the release rate and to establish equilibrium condition. To ensure solubilization of poorly soluble resveratrol, 10 mL of ethanol was added to the system and mixed thoroughly on a magnetic stirrer. Separate tubes were used for each time. At each time point, 500 μL release media was replaced with the same volume of fresh release media. At selected time intervals, loaded halloysite were separated by centrifugation. The supernatant was collected, diluted with ethanol-water (1:1) towards the calibration range. The resveratrol content in the supernatant was determined spectrophotometrically (UV-Vis Spectrophotometer Varian Cary 300 Scan) at 306 nm. Complete release was checked after 1 h sonication of the halloysite sample at the end of each release study. Halloysites devoid of resveratrol were used as a control. From a standard calibration curve of absorbance of resveratrol in supernatant compared to the initial resveratrol amount in the halloysites a concentration profile was derived and plotted against time.

2.5. Release Control with LbL Coatings

Halloysite nanotubes were coated by traditional LbL technique procedure to provide additional control over the drug release rate. Negatively charged halloysite tubes were used as a template for the first step adsorption of cationic polyelectrolyte, PAH or PRM. At the following step, an anionic polyelectrolyte PSS or DXS were deposited. PAH and PSS solutions were prepared in 0.5 M NaCl at the concentration of 2 mg · mL^{-1}, while PRM and DXS solutions were prepared in 0.1 M NaCl at the concentration of 2 mg · mL^{-1}. The assembly was performed at pH 6.5 in order to achieve strongly charged species for all the polycations and polyanions used; so the pH of the polymer solutions was adjusted to 6.5 by addition of HCl/NaOH. For each incubation, the dispersion was continuously shaken for 10 min and the excess of polyelectrolyte was removed by three centrifugation/washing steps with distilled water. The procedure was repeated three times for each polyelectrolyte resulting in the deposition of six polyelectrolyte layers on the halloysite nanotubes. The assembly of polyelectrolyte layers on halloysite tubes was monitored by controlling the surface ζ-potential alternation after deposition of each layer using a Nano ZS90 (Malvern Instruments, UK).

2.6. Efficiency of Resveratrol Loaded-halloysite

To validate the efficiency of resveratrol loaded-HNTs they were used to treat MCF-7 cells because this fitoalexin acts on estrogen receptor expressed on nuclear membrane of this cell line. MCF-7 cells were growth at 37 °C in a humidified atmosphere (95% air and 5% CO_2), Dulbecco's Modified Eagle Medium (DMEM) supplemented with phenol red, 3% L-glutamine, penicillin (100 UmL^{-1}), gentamycin (100 mg mL^{-1}), and 10% fetal bovine serum. Briefly, cells were seeded approximately 50 000 cells/well into a flat bottomed 24-well plate. Resveratrol loaded-halloysite was added at different concentration. For positive control some untreated halloysite were used. After certain time of incubation (24, 48, 72, 96 h) a MTT test was performed. This technique measures the activity of living cells via mitochondrial dehydrogenase activity. The key component is 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazane crystals which are insoluble in aqueous solutions. After an appropriate incubation period, culture was removed from incubator and a MTT solution in an amount equal to 10% of culture volume was aseptically added. Culture was returned to incubator for 3 h. After the incubation period, culture was removed from incubator, the medium was aspired and the formazan crystals have been dissolved in DMSO; pipetting up and down was required to completely dissolve the crystals. The resulting purple solution is spectrophotometrically measured at wavelength of 570 nm.

Another MTT test was performed to test the efficiency of LbL technique used to create halloysite closed at the end to control the release rate. Also in this case MCF-7 cells were growth in the same condition of the previous experiment. Halloysite alone, halloysite coated with PAH/PSS and PRM/DXS, halloysite loaded with resveratrol and coated with the two couple of polyelecrolytes were used to prepare the MTT test. Cell viability was checked at 24 h.

3. Results and Discussion

The chosen drug is poorly soluble in water, the maximal solubility of resveratrol in water is 0.3 mg/mL. According to our studies,[31] we performed loading procedure from aqueous solvent containing ethanol, which provided higher solubility for the drug. Loading of the halloysite nanotubes with resveratrol was based on vacuum cycling of halloysite suspension in saturated solution containing resveratrol, as it was described earlier.[28,33] The air located inside the tubes was replaced by the drug solution during this process (Scheme 1). This cycle was repeated more times in order to get highest efficiency of loading.

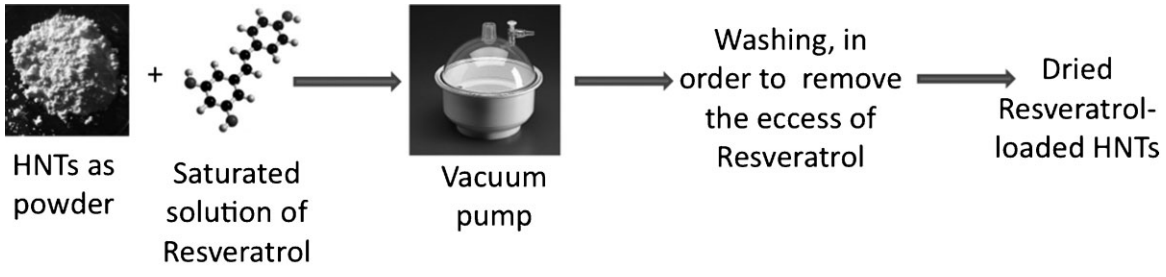
We have performed loading procedure with both different percentage of ethanol in water and with different concentration of resveratrol solution; we obtained best result when we used an aqueous solvent containing 70% of ethanol and with a saturared solution of resveratrol with a concentration of 0.5 M. After loading, halloysite were washed in order to remove any loosely attached substance from the external walls. Evaporation of the solvent under vacuum elevates the concentration of the loading substance. The concentration gradient plays a major role in loading tubes, therefore fast drying solvents with low viscosities such as acetone or ethanol are preferable for organic substances. A high dielectric constant of the solvent is also desirable since it provides ionization of the hydroxyl groups at external and internal walls of the halloysite tubes, which stabilizes halloysite in the suspension and enhances loading of the negatively charged molecules by means of electrostatic force (inner lumen of the halloysite has positive charge at pH of the solution below 8.5).[33,34]

According the formula

$$EE\,(\%) = C_{\mathrm{HNTs}} = (V_{\mathrm{i}}C_{\mathrm{i}} - V_{\mathrm{s}}C_{\mathrm{s}})/V_{\mathrm{HNTs}} * 100$$

we have calculated an efficiency entrapment of resveratrol of about 99.7%, starting from 0.5 M resveratrol solution prepared in water with 70% of ethanol. Higher loading efficiency of the drug can be related to a number of reasons; higher solubility of the drug at a low pH, increased positive charge of the halloysite at inner lumen, etc. The type of used solvent also affects the loading efficiency of the drugs. The ethanol – water mixture provides a better loading efficiency than just pure water, which is related to the higher solubility of the drugs in ethanol. In all of the cases higher loading efficiency leads to faster release.[31,34]

In Figure 2, extended release profile of resveratrol from halloysite nanotubes in buffer solution is elucidated. Controlled release is an attainable and desirable characteristic for drug delivery systems. The factors affecting the drug release rate revolve around the structure of the matrix



Scheme 1. Scheme of Resveratrol-loading inside HNTs ref.[30]

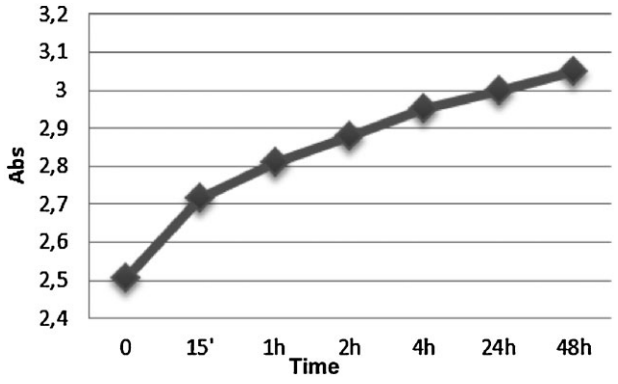


Figure 2. Resveratrol release profile from halloysite clay nanotubes. Concentration (in mg\mL) of resveratrol released by HNTs plotted versus time as calculated through a standard calibration curve by absorption spectroscopy.

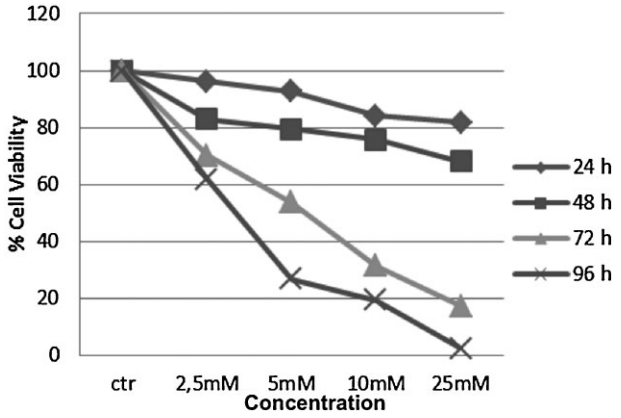


Figure 3. MTT test for cell viability of MCF 7 cells cultured for 24, 48, 72, 96 h in presence of the indicated concentration of resveratrol-loaded halloysite.

where the drug is contained and the chemical properties associated with both the clay and the drug. The drug release is also diffusion controlled as the drug can travel through the pores of halloysite. The most desirable release profile would show a constant release rate with time. However, in many cases release profiles are more complicated and often contain two main expulsion processes: the first being an initial burst of expelled medication from the halloysite surface; the second, a usually more constant stage with release rates dependent on diffusion and degradation.[35]

As one can see the complete release of resveratrol happens within 48 h. An initial burst is observed within 15 min followed by a prolonged release. About 20% initial bust release is typical for different molecules loaded into the clay nanotubes, and may be due to the fast dissolution of the material stored in tube fines and in the natural surface pocket and the end of the loosely rolled surface clay sheet. It is possible to remove much, but not all of the adsorbed active agent from external surface of the cylinders. Multiple centrifugations were used for the removal of exogenous material.

To validate this new delivery system allowing for sustained killing of MCF-7 cells, we loaded halloysite with resveratrol 0.5 M in water/ethanol solution. Nearly 100% drug-encapsulation efficiency was determined by UV-Vis absorbance analysis under such experimental condition, we perform a MTT test to verify if resveratrol was released from the lumen of halloysite and carry out its apoptotic action on MCF-7 cells. MCF-7 cells are ERα positive so resveratrol exhibited apoptotic cell death, suggesting that apoptosis induction could be ERα mediated. As we can see in Figure 3 cell viability was estimated at 24, 48, 72 and 96 h under different resveratrol concentrations: 2.5, 5, 10, $25 \cdot 10^{-3}$ M, estimated with calibration curve obtained with standard solution of resveratrol. Cell viability decrease in concentration and time dependent manner. At 24 h one can see that resveratrol has not yet been fully released, cell viability remains high, around 80%, even with the highest concentration used ($25 \cdot 10^{-3}$ M). In prolonged treatment cell viability decrease enormously as shown 72 h and 96 h trend curves, the percentage of cell survived reached 5%. Resveratrol could be either an agonist or an antagonist of the ER α, depending on the cellular context and concentration.[36] Therefore, the ability of resveratrol to bind the ERα and to modulate its activity could help explain the specific effects of this molecule on cell proliferation and apoptosis in human breast cancer cells.

Halloysite nanotubes can be loaded with a wide range of molecules followed by their retention and slow release. However additional functionalization is required for controlling the release rate, because physiological environment lead to fast releasing of substances.

As already know halloysite nanotubes have a negative surface, which will not necessary prevent cellular uptake. They can be effectively coated by sequential adsorption of positively and negatively charged polyelectrolytes according to the layer by layer technique (LbL). Polyelectrolytes form a shell around halloysite, including tube ends, and provide a diffusion barrier which slows down the release.[4]

After loading protocol, halloysite was coated with two different couple of polyelectrolytes: PEI-PSS/PAH and PRM/DXS (Figure 4). LbL encapsulation was monitored by measuring the ζ-potential of the halloysite and the alternation of negative and positive surface potential indicated effective encapsulation. The first polymer couple is biocompatible but no biodegradable so it forms a stable coating around the halloysite also after internalization by cells; the second one is biocompatible and biodegradable so when halloysite interact with cells, the intracellular proteases digest the polyelectrolytes, and resveratrol is released.

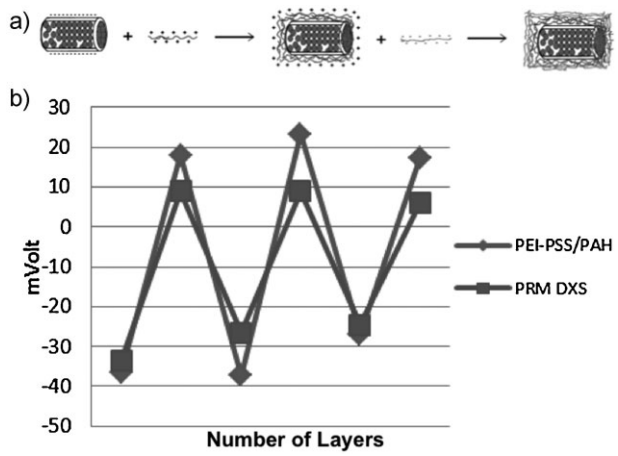


Figure 4. Schematic of LbL deposition of the polyelectrolytes (a); alternation of the halloysite ζ-potential during LbL process with synthetic and not biodegradable couple of polyelectrolytes (blue line) and biocompatible and biodegradable couple (red line).

To check this hypothesis a MTT test was performed using halloysite loaded with resveratrol and coated with PEI-PSS/PAH and halloysite loaded with resveratrol and coated with PRM/DXS; as controls were used halloysite alone, tubes coated with PEI-PSS/PAH and with PRM/DXS. As demonstrated in the Figure 5 both halloysite alone and halloysite coated with the two couple of polyelectrolytes did not decrease cell viability, confirming that these systems are not toxic for cells, and they can be used as drug delivery system. Resveratrol-loaded and coated with PEI-PSS/PAH halloysites have no cytotoxicity because this couple of polyelectrolytes are not enzymatically degraded by proteases, so the resveratrol remain entrapped inside the lumen of our carrier, and it is not able to carry out its apoptotic activity. On the contrary, resveratrol loaded and coated with PRM/DXS halloysite have an apoptotic effect because these two polyelectrolytes were spontaneously degraded

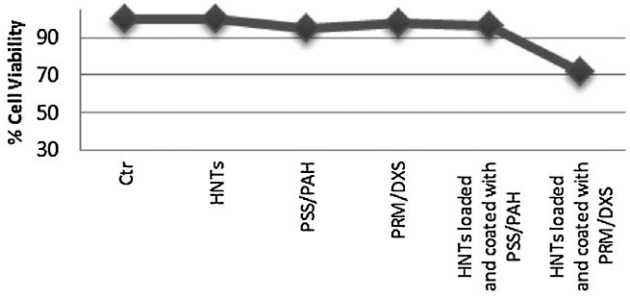


Figure 5. MTT test for cell viability of MCF 7 cells cultured for 24 h in presence of halloysite alone, halloysite coated with PSS/PAH and with PRM/DXS, resveratrol loaded halloysite coated with PSS/PAH and with PRM/DXS.

under physiological conditions; in this case resveratrol inside halloysite has been slowly released and it kills MCF-7 cells as expected.

Thinking about a future clinical application of drug-loaded HNTs one may imagine that their fate inside human body could be an issue. Instead since such clays are not degradable one possible destiny of such nanocarriers, after having released their drug content, could be an accumulation in the liver. However due to their small size, one may also think that they will be naturally removed from the body and that should have a limited side effects in terms of inflammation or possible kidney stones formation. Any other concern regarding possible heavy metal contamination has been solved by a very high purification procedure performed by Applied Minerals Inc. at the time of HNTs production.

4. Conclusion

Resveratrol is a polyphenolic compound possessing antiproliferative properties, and other biological activities but its usage is limited because of low solubility in water. In view of its importance in medical applications it is necessary to encapsulated it inside a nano-delivery system. 50-nm diameter halloysite nanotubes can be used as drug delivery system because of their biocompatibility and allowing drug sustained release. These tubes can be loaded with a wide range of compounds following their retention and slow controlling the release with further surface modifications, including the end-stopper formation.

Halloysite clay can be considered as green and natural nanocarriers for hydrophobic drugs encapsulation. Resveratrol-loaded clay nanotubes showed slow 48-hour release with a constant release rate with time. An additional functionalization for controlling the release rate was made using layer-by-layer polyelectrolyte multilayer coating onto the tube external surface and further decrease of release rate was demonstrated. Based on these results, halloysite clay nanotubes are proposed as novel natural pharmaceutical carriers.

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