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Dispersion of titanate nanotubes for nanomedicine: comparison of PEI and PEG nanohybrids

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In the present study, we report the dispersion of titanate nanotubes (TiONts) *via* polymer grafting (PolyEthylene Glycol, PEG) or polymer adsorption (PolyEthylene Imine, PEI) where different TiONts/polymer ratios have been investigated. The TiONts/PEI and TiONts/PEG nanohybrids were characterized by Scanning and Transmission Electron Microscopy as well as by zeta potential measurements in order to determine both their dispersion state and stability in water (at different pH for zetametry). The nature of the chemical bonds at the surface of these nanohybrids was investigated by Fourier-Transformed InfraRed (FTIR) spectroscopy while the grafting densities of PEG on the nanotubes were quantified by ThermoGravimetric Analyses (TGA). The nanohybrids reported here are promising tools for biotechnology applications due to their tubular morphology, their very good dispersion in water and the reactivity of their surface.

Introduction

In the last decade, titanate nanotubes (TiONts) have generated a great deal of interest for their potential applications. In 1998, Kasuga *et al.* performed the first synthesis of this material by hydrothermal treatment.[1] Since this pioneering work, research has continually progressed in order to better understand their physico-chemical properties and their formation mechanisms. TiONts have been studied for a variety of applications from photocatalysis,[2] to ion exchange,[3, 4] photochemistry[2] and electrochemistry.[5] They are now a surface and material of interest for biomedical applications such as dopamine detection,[6] bone regeneration,[7] molecule vectorization,[8, 9] vectorization for imaging when conjugated with SPIO[10] and cancer cell radiosensitization.[11] An interesting feature of the TiONts, particularly for drug delivery, is its shape as it has been shown that tubular nanoparticles have a higher internalization by cells than their spherical counterparts.[12] Most of these bioapplications require these nanomaterials to be functionalized. Functionalization enables the TiONts to both carry therapeutic molecules and also improves their stability for vectorization applications. Indeed, TiONts synthesized by hydrothermal treatment are strongly agglomerated and require dispersion prior to any bio-application. TiONts are negatively charged at physiological pH due to the presence of hydroxyl groups on their surface above their isoelectric point (pH 3.7).[10] Consequently, they are able to react with a wide range of functional molecules.

Surface functionalization of TiONts has been studied by various approaches in literature. They were first functionalized with polystyrene (to reinforce the polymer mechanical properties) using allyltriethoxysilane or *n*-propyltriethoxysilane as anchoring agents,[13] with 2,6-dihydroxyantraquinone (anthrafavic acid) to improve the photoelectrochemical generation of hydrogen from water,[14] with methyl methacrylate (MMA) by *in situ* atom transfer radical polymerization,[15] later on, they were coated with the thermo-responsive poly(*N*-isopropylacrylamide) (PNIPAAm),[16] with PolyEthylenImine (PEI) as an efficient carbon dioxide adsorbent[17] and Fe_3O_4-TiONts composite were functionalized with C_{18} and alginate polymer cage (called ALG@C_{18}-Fe_3O_4-TNs) for environmental purification purposes.[18] Titanate nanotubes have been functionalized with long-chain amines during their hydrothermal treatment by the addition of dodecylamine and octadecylamine.[19] The surface of titanate nanobelts have been modified with poly(allylamine hydrochloride) for nonlinear optical investigations.[20] Recently, titanate nanotubes have also been functionalized with APTES (3-aminopropyltriethoxysilane) to improve compatibility between polymer matrix and nanofillers[21, 22] or with AAPTS ([1-(2-

amino-ethyl)-3-aminopropyl]trimethoxysilane) for the removal of Cr(VI) from aqueous solutions.[23] With the same end goal (purification from hexavalent chromium compounds), Nui *et al.* published an environment friendly alternative to amino-functionalize titanate nanotubes using an S_N2 reaction starting from 2-bromoethylamine hydrobromide.[24]

To the best of our knowledge, very few studies have so far focused on the functionalization of titanate nanotubes for biomedical applications. For instance, Shi *et al.* have reported the coating of titanate nanotubes with the biodegradable Poly(ε-CaproLactone) (PCL)[25] or TiONts inclusion into biodegradable polyfumarate photopolymers (PPF:DEF) for putative applications in tissue engineering or regenerative medicine.[26] However, the potential of its shape for drug delivery purposes seems evident, as mentioned earlier.

PEGylation increases the dispersion and thus the stability of nanoparticles, while providing stealth properties against the reticuloendothelial system by decreasing protein adsorption; consequently it decreases clearance rate of nanoparticles.[27] In addition, it has been reported that PEGylation improves intracellular trafficking of nanoparticles in the cytosol.[28] PEGs are biocompatible and biodegradable polymers used in a wide range of molecular weights (usually from 160 to 20,000 $g.mol^{-1}$ for bio-applications).[29] Metal oxide particle PEGylation with silanated methoxy-PEG is a widely used and relatively simple procedure.[30]

Polyethylenimine (PEI) is a macromolecule that is well known as a gene delivery carrier.[31] Among established commercial transfection agents, jetPEI® (Polyplus Transfection™) includes this polymer as a part of its composition. The main advantage of PEI is its proton sponge effect (recently debated),[32] which would permit a direct access to the cytosol from endosomes after cellular internalization. Indeed, PEI-DNA complexes are internalized by endocytosis, followed by the proton sponge effect of the polymer (or an interaction between the polymer and the vesicle membrane), which ruptures the vesicle membrane and delivers the complex to the cytosol.[33] Thus, PEI indirectly improves the intracellular trafficking of DNA. PEI also permits the reversal in charge of negatively charged materials, as it is the case for TiONts at physiological pH.[9] This might be beneficial to increase interactions of nanohybrids with cells.

All these reasons taken together make PEG and PEI excellent candidates to disperse TiONts and were the rationale for the design of the current study.

Herein we provide the first example of modified TiONts with PEG, the gold standard of functionalization for the stability and dispersion of nanobiocarriers. PEG is a neutral polymer leaving unchanged the negative charge of the TiONts, PEI was then selected as a second option for dispersion, utilizing its charge reversal capability on negatively charged surfaces and its proton sponge effect. Consequently, the TiONts/polymer hybrids as prepared were either positively or negatively charged, dispersed and biocompatible.

Experimental

Chemicals: Titanium dioxide (TiO_2) rutile precursor was purchased from Tioxide. Sodium hydroxide (NaOH), hydrochloric acid (HCl 37%), methoxy-PEG (molecular weight 550 and 2000 g/mol), 3-Isocyanatopropyltriethoxysilane (ICPTS), 3-Aminopropyl)triethoxysilane (APTES), Tetrahydrofuran (THF), dibutyltin dilaurate (DBTL), *n*-hexane, polyethylenimine (molecular weight M_n 1800 g/mol) and rhodamine B isothiocyanate (RITC) were purchased from Sigma-Aldrich and used without any further purification. THF was distilled over sodium metal and benzophenone just before use.

Titanate nanotube synthesis: Titanate nanotubes were prepared by a classical hydrothermal method adapted from.[34] Briefly, TiO_2 rutile precursor powders (440 mg) were added to a NaOH aqueous solution (10 mol/L, 110 mL).[35] The mixture underwent ultrasounds (30 min, 375 W, Sonics Vibra-Cells) before being transferred into a sealed Teflon reactor under magnetic stirring. The hydrothermal reactor temperature was kept at 150°C for 36 hours. The resulting white product was isolated by centrifugation and washed with deionised water until pH 6. Finally the powder was freeze-dried before characterization.

Preparation of TiONts/PEI nanohybrids: TiONt suspension (2 $mg.mL^{-1}$) and PEI_{1800} solution (73.1 $mg.mL^{-1}$) were prepared and homogenized in an ultrasonic bath (Branson 5200, 30 min). For TiONts/PEI (1:1, w:w), 100 µL of TiONt suspension and 2.74 µL of PEI_{1800} solution were mixed together in a 1.5 mL Eppendorf, the volume was completed to 500 µL with HCl and deionised water to adjust the pH to 7.4. Quantities were adapted for other nanohybrids. The final preparation for each nanohybrid was subjected to vortex (maximal speed) for 15 min then left on a gyro-plate for 12 h.

Preparation of TiONts/PEG nanohybrids: To functionalize TiONts with mPEG (mPEG-Si-TiONts), silanated methoxy PolyEthylene Glycol ($mPEG_{550}$–Si or $mPEG_{2000}$–Si) was synthesized as described elsewhere.[36] Briefly, 3-isocyanatopropyltriethoxy silane was attached to mPEG, using dibutyltindilaurate, at 60°C in dried THF under nitrogen flow for 48 h. mPEG-Si was precipited in *n*-hexane. Subsequently, 50 mg of TiONts were introduced into a schlenk containing 10 mL of an ethanol/water mixture (50:50, v:v, adjusted to pH 4) and were sonicated for 15 min. In parallel, 1 mg of m-PEG_{550}-Si or 1500 mg of m-PEG_{2000}-Si were dissolved in 10 mL of ethanol/water mixture (50:50, v:v, pH 4.0) under sonication before being added to the schlenk. The total volume of ethanol/water is supplemented to 30 mL and sonicated once more. After 24 h at 21°C the reaction mixture was first centrifuged (25,000 ×g, 15 min, 20°C) then dialyzed in deionized water (3.5 kDa MWCO dialysis tubing) for 3 days to remove any free PEG. Finally, the TiONt suspension was freeze-dried before storage.

Preparation of rhodamine (RITC) functionalized TiONts: TiONts have been functionalized with RITC in order to detect the nanotubes internalization in cells by flow cytometry.

Subsequently, TiONts/RITC have been functionalized with PEI following the above-mentioned protocol ("Preparation of TiONts/PEI nanohybrids"). Briefly, TiONts have been first of all silanized with APTES: 50 mg of TiONts were introduced into a Schlenk containing 60 mL of an ethanol/water mixture (50:50, v:v, adjusted to pH 4) and were sonicated for 30 min. After 72 h at 21°C the reaction mixture was dialyzed in deionized water (3.5 kDa MWCO dialysis tubing) for 5 days to remove any free APTES. The TiONts/APTES suspension was freeze-dried before being conjugated to RITC. 11.1 mg of TiONts/APTES were dispersed in 10 mL of deionized water and sonicated for 15 min. Then 100 µL of DMSO containing 1.55 mg of RITC were added using Schlenk's techniques. After 24 h of reaction at 21°C, the TiONts/RITC were dialyzed in deionized water (3.5 kDa MWCO dialysis tubing) for 4 days to remove any free RITC, then freeze-dried.

Characterizations: Nanotube morphology and their agglomeration state were first investigated by Scanning Electron Micrograph (SEM) (JEOL JSM-6400F). Freeze-dried powders were deposited on carbon tapes and observed after carbon coating. Transmission Electron Microscope (TEM) observations were performed on a JEOL JEM-2100 LaB_6 microscope operating at 200kV and equipped with a high tilt pole-piece achieving a point-to-point resolution of 0.25 nm. Powders were dispersed in ethanol before deposition onto a carbon membrane supported by a copper grid. Surface area measurement has been performed using a BELSORP-mini apparatus with N_2 gas adsorption. The BET (Brunauer–Emmett–Teller) method has been used to calculate specific surface area values (S_{BET}) from the isotherm of nitrogen adsorption. The water desorption from nanoparticles and the decomposition of polymers were studied by thermogravimetry (SETARAM TAG24). This symmetric thermobalance is able to measure weight variations of 0.1 µg. Heating rate was 2°C from 25°C up to 650°C under N_2/O_2 atmosphere (120/40 mL/min). Sample weight was around 5-10 mg. Zeta potentials were measured with a Malvern Nano ZS instrument supplied by a DTS Nano V6.20 software. The suspensions of TiONts were prepared in 10^{-2} M NaCl aqueous solutions. The pH of the solution was adjusted from 3 to 11 by addition of HCl or NaOH solutions. FTIR (Fourier Transformed InfraRed) measurements were recorded on a Bruker IFS 28 using OPUS version 3.1. Pellets were made of 2 mg of sample mixed within 200 mg of dried KBr. The PEI spectrum was obtained by ATR (Attenuated Total Reflectance). PEI density of grafting has been determined using UV-visible analysis based on the method of F. Ungaro *et al.*[37]. Briefly, the samples have been centrifuged (25,000 ×g, 25 min) to separate the nanohybrids from the free PEI. Subsequently, the supernatant (10 mL) has been collected and 100 µL of a Cu^{2+} solution (10 mg/mL) has been added. The absorbance of the formed complex has been read at 275 nm.

Cardiomyocyte culture: The primary cultures of neonatal rat cardiomyocytes were obtained as described earlier[38, 39] from 2- to 4-day-old Wistar rat hearts. Briefly, cardiomyocytes were dissociated from ventricular tissue by repeating 10 times 10 min incubation at 37°C in 0.1% trypsin (Difco, Detroit, MI, USA). The dissociated cells were collected and resuspended in Ham's F-10 medium (Lonza, Verviers, Belgium) supplemented with 20% fetal calf serum (Lonza), penicillin (200 $UI.mL^{-1}$; Lonza), streptomycin (150 $UI.mL^{-1}$; Lonza) and $CaCl_2$ (0.135 $mg.mL^{-1}$; Merck). A differential attachment technique was used to selectively enhance the cardiac myocyte density.[39] Cells were then seeded as needed and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced 24 h after seeding and every alternate day.

CHO-k1 culture: CHO-k1 (Chinese Hamster Ovary cells, adherent fibroblastoid cells) were cultured in RPMI supplemented with 10% FBS and 1% penicillin-streptomycin.

Cell viability (MTT Assay): $0.3.10^6$ cells/well (for cardiomyocytes) and $0.1.10^6$ cells/well (for CHO-k1) were seeded in 24-well plates (Falcon Primaria 3802; Becton Dickinson, Oxnard, CA, USA). The reduction of 3-(4,5-dimethyliazol-2-yl)2,5-diphenyltetrazolium bromide (MTT; Sigma) to formazan was used to assess cell viability.[40] Cells were incubated with nanohybrids in serum free media and washed twice with PBS before being incubated for 1 h with MTT reagent (500 µL/well of 24-well plates, 2 $mg.mL^{-1}$ in Puck's medium supplemented with glucose 1.1 $g.L^{-1}$) under normoxic condition. MTT was then replaced by 500 µL of propan-2-ol containing 0.1 N HCl. Absorbance values were finally read at 570 nm using a plate reader.

Internalization study by flow cytometry: Cardiomyocyte cells were seeded in 6 well plates ($1.5.10^6$ cells/well) in Ham's-F10 supplemented medium. Nanotubes were functionalized with RITC and PEI. (RITC-TiONts)/PEI nanohybrids were incubated with cardiomyocytes in serum free media for 5 h before being collected and evaluated by flow cytometry.

Transmission Electron Microscopy: After 5 h of incubation with hybrids, cells were washed and fixed in 4% paraformaldehyde and 1.4% of glutaraldehyde in Sorensen phosphate buffer (0.1 mMol/L, pH 7.4). Then the samples were washed with PBS and incubate with 1% osmium tetraoxide at room temperature for 1 h. Then samples were gradually dehydrated in 30, 50, 70 and 95 % ethanol (each for 15 min) and finally three times with absolute ethanol for 15 min. Sample were embedded in resin overnight at room temperature. Sample slices were obtained using an ultramicrotome before being incubated with uranyl acetate to improve the sample contrast. Observations were done using a HITACHI H-7500 microscope operating at 80 kV.

Statistics: Statistics were performed using the unpaired t-test from GraphPad Prism software.

Results and Discussion

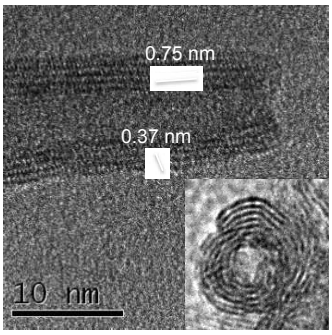


Fig. 1 HRTEM images of a titanate nanotube: longitudinal view with measured inter-reticular distances and cross-section (insert, lower right).

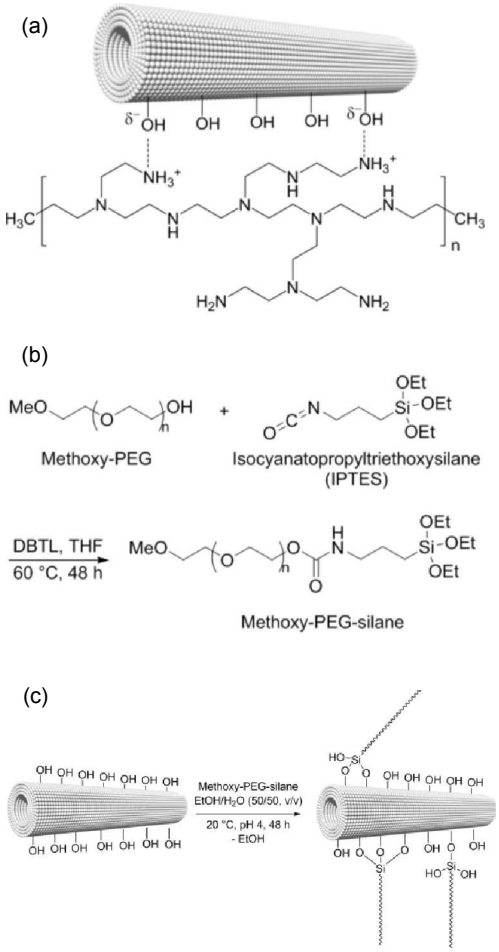
The synthesized titanate nanotubes have been previously described by our group.[35] They are about 10 nm in diameter (**Fig. 1**) and a few hundred of nanometers in length. They are arranged in a spiral configuration (insert in **Fig. 1**, cross section) and their two main inter-reticular distances are 0.75 nm (distance longitudinal to their axis) and 0.37 nm (**Fig. 1** and ref. [35]). This is in agreement with distances reported in literature for titanate nanotubes obtained by hydrothermal treatment.[41] Their high specific surface area (here about 121 $m^2.g^{-1}$ measured by BET (Brunauer–Emmett–Teller)) represents a tunable surface with straightforward functionalization options offered by their hydroxyl groups.[35] Indeed, two functionalization alternatives can be used: the electrostatic functionalization with cationic polymers *versus* the covalent approach.

Table 1 Surface characterizations and stability profile of the nanohybrids: grafting density of polymer (PEG_{550}, PEG_{2000} or PEI_{1800}) on TiONt surface and zeta potential at physiological pH of the resulting nanohybrids (w:w ratios).

Nanohybrids	**TiONts/ PEG_{550}**	**TiONts/ PEG_{2000}**	**TiONts/ PEI_{1800} (1:0.1)**	**TiONts/ PEI_{1800} (1:1)**	**TiONts/ PEI_{1800} (1:10)**
Polymer interaction	Covalent		Electrostatic		
Theoretical* (experimental) grafting density (Polymer(s)/nm^2)	0.18 (0.10)	74.7 (1.30)	0.28 (0.16)	2.8 (0.42)	28 (nd)
Zeta potential (mV) at pH 7.4 in NaCl 10^{-2} $mol.L^{-1}$	-27.0 ± 1.5	-25.1 ± 0.2	-2.0 ± 0.7	39.0 ± 0.6	39.0 ± 1.0

* Detailed calculation is available in Supporting Information SI1-2. nd: not determined.

We have selected PEI (electrostatic functionalization) and PEG (covalent functionalization) for their wide interest, respectively in DNA vectorization and drug delivery. Nanohybrids are obtained by (i) charge interaction between the hydroxyls of TiONts and the amines of the PEI (**Scheme 1a**) or by (ii) the covalent binding between the hydroxyls of TiONts and the silanes of a methoxy-PEG-silane (**Scheme 1b-c**). The calculated and experimental densities of polymer on the nanotubes are reported in **Table 1**.



Scheme 1 (a) Branched PEI (n=1800) adsorption on titanate nanotubes at physiological pH, (b) Si-mPEG synthesis and (c) its grafting on titanate nanotubes.

Experimental densities vary from 0.1 to 1.3 polymers per nm^2 of particles depending on the polymer and grafting conditions. Grafting densities have been determined using thermogravimetric analysis (TGA) for PEGs (**Fig. 2**) and UV-vis spectrophotometry for PEIs. The PEG mass loss due to its decomposition has been considered between 125°C and 650°C to exclude the contribution of adsorbed water. Calculation details and additional data (TiONts/PEI from 1:0.01 to 1:10 ratios) are available in Supplementary Information SI1-2.

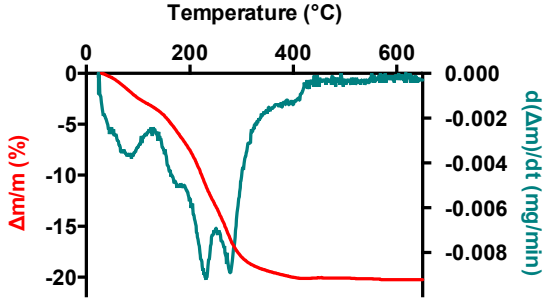


Fig. 2 TGA with derivative weight of $TiONts/PEG_{2000}$ from room temperature to 650°C.

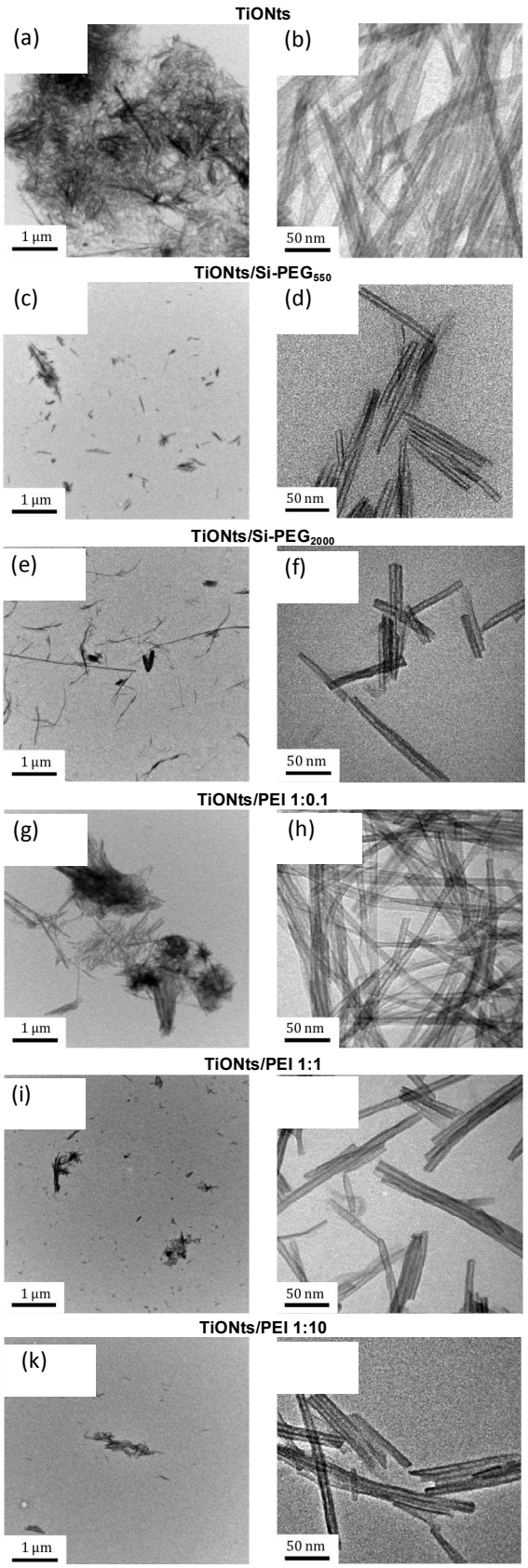


Fig. 3 TEM images of (a-b) bare TiONts, (c-d) TiONts/Si-PEG_{550}, (e-f) TiONts/Si-PEG_{2000}. TiONts/PEI_{1800}: (g-h) 1:0.1, w:w, (i-j) 1:1, w:w, (k-l) 1:10, w:w.

After their synthesis and lyophilization, titanate nanotubes are strongly agglomerated and the size of agglomerates can reach several microns (**Fig. 3a and SEM in SI3**) of tightly packed tubes (**Fig. 3b)**. Their functionalization is then critical, but also multifold, as beyond their improved circulation time (using PEG)[27] and intracellular trafficking (using PEI[33] and PEG[28]), the polymer enhances the particle stability and their dispersion state. Indeed, the use of PEG as dispersant seem to enable tube individualization (**Fig. 3c-3f and SI3)** compared to bare nanotubes (**Fig. 3a, 3b**), while keeping the surface charge of TiONts unchanged and negative (**Fig. 4**). In parallel, PEI caused a gradual increase in the level of TiONt dispersion with increasing amounts of the polymer (from 1:0.1 to 1:10 TiONts/PEI w:w ratios) (**Fig. 3g-l and SI3**). We observed a greater proportion of agglomerates under the micrometer size and individualized tubes beyond a 1:1 ratio of PEI on TiONts (**Fig. 3i-3l**). This result has been correlated with zeta potential measurements, at varying pHs, that would indicated that TiONts/PEI 1:1 and 1:10 significantly increased nanotube surface charge in the range of pH 2 to 9 (**Fig. 4**) promoting electrostatic stabilization compared to the 1:0.1 ratio. Indeed, their zeta potential absolute values are significantly higher (*ca.* 40 mV) than the 1:0.1 TiONts/PEI hybrids (*ca.* 10-30 mV).

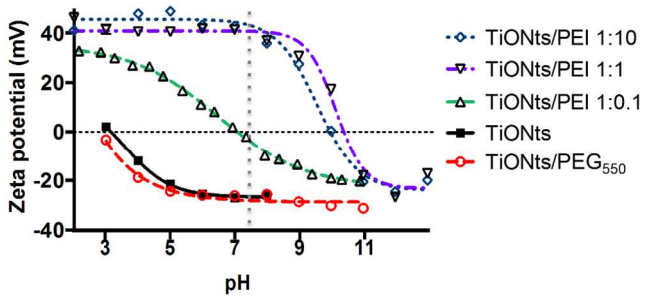


Fig. 4 Zeta potential versus pH of bare TiONts, TiONts/PEI_{1800} and TiONts/PEG_{550} nanohybrids as a function of pH (in NaCl 10^{-2} mol.L^{-1}).

Bare TiONts are negatively charged at physiological pH (due to their hydroxyl groups)[35] and PEI was able both to neutralize the tube surface (1:0.1 ratio) and to reverse their charge (1:1 and 1:10 ratios) at pH 7.4 (**Fig. 4**). The surface charge was not statistically different between 1:1 and 1:10 ratios in the range of pHs that are of interest for vectorization studies (*i.e.* pH 5-7.4). Thus, the TiONts/PEI 1:1 nanohybrid has been selected for further physico-chemical characterization studies. The isoelectric point of TiONts shifted from about 3 (bare tubes and TiONts/PEG_{550}) to 7 (TiONts/PEI 1:0.1) and 10 (TiONts/PEI 1:1 and 1:10) (**Fig. 4**). Finally, we investigated the stability of suspensions by following their absorbance at 600 nm and pH 7 over 150 minutes (**Fig. 5**). Unfunctionalized TiONts settled quickly while polymer-coated TiONts showed greater stability, the best suspension stability being exerted by TiONts/PEI_{1800} (1:1). This correlates with zeta potential measurements where higher absolute values of zeta potential could be further indicative of a greater stability of the TiONts/PEI (1:1) suspension (**Table 1**, **Fig. 4**).

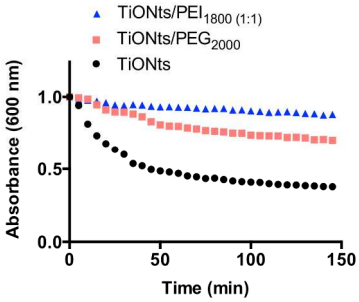


Fig. 5 Stability of TiONts, TiONts/PEG_{2000} and TiONts/PEI_{1800} suspensions over 150 min following their absorbance at 600 nm and pH = 7.

The presence of bound polymer to the titanate nanotubes has been validated by Fourier transformed infrared spectroscopy (FTIR) (**Fig. 6**) as previously shown for other PEGylated particles in literature.[42, 43] Indeed, the following vibrations on the TiONts/Si-mPEG_{2000} absorbance spectra confirmed the surface PEGylation: 2887 cm^{-1} (-CH_2 stretching), 1720 cm^{-1} (C=O stretching) and 1110 cm^{-1} (C-O-C stretching) were found to be shared by both Si-mPEG_{2000} and TiONts/Si-mPEG_{2000} nanohybrid spectra while missing from TiONt spectrum (**Fig. 6**, highlighted by stars). Similarly, PEI vibrations assigned as 2962 cm^{-1} (C-H stretching), 2844 cm^{-1} (C-H stretching), 1565 cm^{-1} (N-H bending)[44] 1315 cm^{-1} (-CH_2 wag) and 1110 cm^{-1} (C-N stretching)[45] were present on both PEI_{1800} and PEI_{1800}-TiONt spectra while missing from TiONt spectrum (**Fig. 6**, highlighted by double stars). The presence of polymers on TiONt surface has also been proven by XPS analyses. Carbon and nitrogen atomic concentrations increase because of PEG or PEI graftings (data not shown).

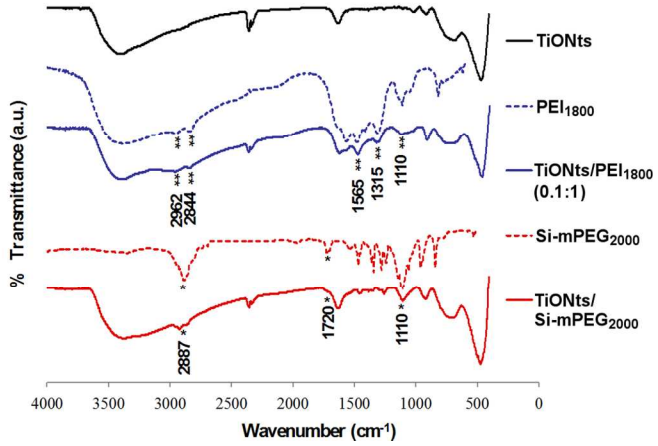


Fig. 6 FTIR spectra of bare TiONts, PEI_{1800}, TiONts/PEI_{1800} (1:1, w:w) nanohybrid, Si-mPEG_{2000} and TiONts/Si-PEG_{2000} nanohybrids.

The cytotoxicity of TiONts and TiONts/PEI_{1800} with regard to neonatal rat cardiomyocyte cells has been previously reported by our groups and shown to be non-cytotoxic after a 24 h period of incubation (*i.e.* viability greater than 90%).[9] Subsequently, TiONts/PEG_{2000} cytotoxicity towards cardiomyocytes has also been evaluated and has not demonstrated any change in cell survival below 5 µg/mL TiONts/PEG_{2000}, compared to untreated cells (**Fig. 7**). However, some degree of cytotoxicity (*i.e.* a cardiomyocyte viability of 78%, p=0.0177 *vs.* control) has been observed after a 24 h period of incubation with 10 µg/mL TiONts/PEG_{2000} (**Fig. 7a**).

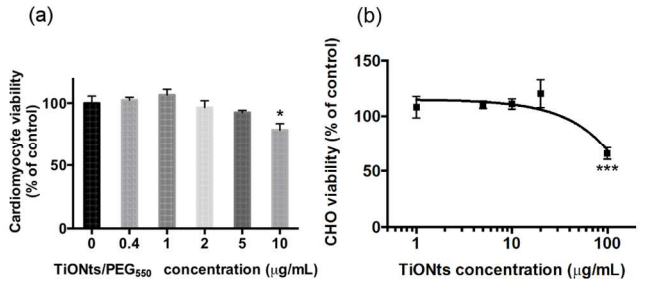


Fig. 7 Cell viability assays (MTT) of (a) cardiomyocyte viability after incubation with various concentrations of TiONts/PEG_{2000} and (b) CHO cell after incubation with a dose range reaching 100µg/mL during 24 h in their respective serum deprived media. Significance was indicated by: *p < 0.05 and ***p < 0.001.

A second cell line has been selected (CHO-k1 cells) to further evaluate the cytotoxicity induced after 24 h of incubation with TiONts. These cells did not show any sign of cytotoxicity below 20 µg/mL of TiONts (**Fig. 7b**). This suggests that titanate nanotubes do not induce toxicity in the range of concentrations that would potentially be utilized for vectorization studies (*i.e.* significantly below 10 µg/mL). The molecular weight of TiONts is not a trivial parameter to define as the formula of these nanotubes can be defined as $Na_yH_{2-y}Ti_nO_{2n+1},xH_2O$.[35] Minimally, 10 µg/mL of $NaHTiO_3$ (MW = 119.8 g/mol) represents a very high concentration of TiONts (83.5 µM).

The higher the molecular weight of $Na_yH_{2-y}Ti_nO_{2n+1}$, xH_2O, the higher the molar concentration increases. This suggests that TiONts are safe nanovectors *in vitro* since they display a non-cytotoxic profile till 20 µg/mL (*i.e.* up to 167 µM considering $NaHTiO_3$ formula).

An internalization study has been carried out in order to further highlight that TiONts can be utilized as potential nanocarriers. They first have been functionalized with a fluorescent dye (rhodamine isothiocyanate, RITC), a small detectable molecule that substitutes a drug for the present proof-of-concept. RITC has been covalently attached to the surface of the nanotubes (reaction between RITC and amino-silanes modified TiONts) and this was helpful in detecting TiONts. The loading of RITC on nanotubes was 38 µg per mg of TiONts (measured by fluorescence detection and referenced to RITC standard curve). Subsequently, different (RITC-TiONts)/PEI hybrids have been prepared with 1:1, 1:10, 1:20 and 1:30 ratios (TiONts:PEI w:w ratios) and both the ratio (*i.e.* the PEI loading on the tubes) and TiONt concentration (0.8, 4, 8 and 16 µg/mL of TiONts) effects on the internalization efficiency have been assessed. RITC allowed us to track the hybrids both by fluorescence microscopy (**Fig. 8a**) and flow cytometry (**Fig. 8b**). The internalization of the different hybrids followed the same trend with increasing TiONt concentration (**Fig. 8b**). After 5 h of

incubation, about 16% of cardiomyocytes have internalized (RITC-TiONts)/PEI hybrids at 0.8 µg/mL concentration, then about 70%, 77% and 74% cells have internalized hybrids with the respective concentrations: 4, 8, 16 µg/mL.

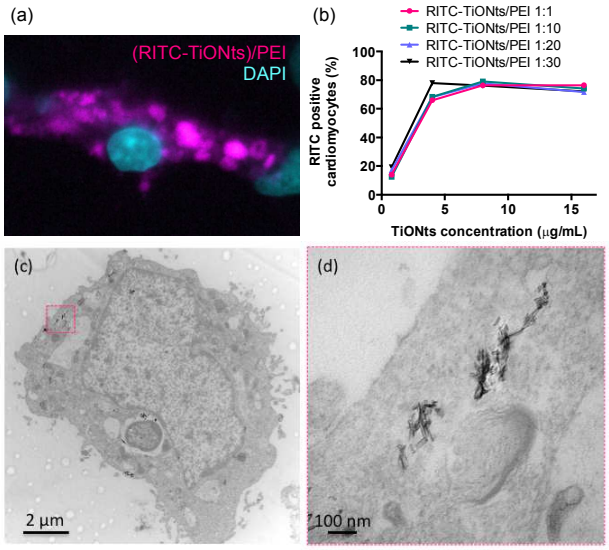


Fig. 8 Internalization study of (RITC-TiONts)/PEI in cardiomyocyte cells after 5 h of incubation. (a) Fluorescence microscopy image illustrating the RITC conjugated hybrids in cardiomyocytes cells, (b) internalization of the nanohybrids depending on the TiONt concentration and polymer ratio assessed by flow cytometry, (c) Transmission Electron Micrograph and zoomed area (d) of TiONts/PEI in cardiomyocyte cells that visually confirm their internalization.

The pattern clearly showed that the hybrid uptake was governed by the nanotube concentration and not by the polymer coverage on their surface (**Fig. 8b**). Transmission Electron Micrographs of cardiomyocytes confirmed TiONt internalization and nanotubes could be located in vesicles (**Fig. 8c**) as well as inside the cytosol (**Fig. 8d**).

Conclusions

Titanate nanotubes have been functionalized with polyethyleneimine and polyethylene glycol for potential translation towards biomedical applications (such as vectorization, drug delivery or transfection). Their high specific surface area (121 $m^2.g^{-1}$ compared to S_{BET} of spherical rutile TiO_2 (Tioxide®) which is 7 $m^2.g^{-1}$) can display a negative, neutral or positive surface charge at physiological pH once chemically modified with a neutral or a charged polymer (PEG or PEI respectively). This makes the surface tuneable for various applications. These polymers were able to significantly disperse the nanotubes and their grafting and adsorption at the TiONt surface have been confirmed by zeta potential measurements, TGA and FTIR. Finally, the functionalized nanotubes did not induce cytotoxic response with regard to neonatal rat cardiomyocyte cells and CHO-k1 (except a light cytotoxicity at high concentrations). In addition, an internalization study has shown a dose dependence internalization of TiONts/PEI nanohybrids that was driven by TiONt concentration. An increased loading of PEI on their surface did not modify the cardiomyocyte uptake of the hybrids. This study demonstrates that the tuneable surface of titanate nanotubes make them potential non-cytotoxic carriers for *in vitro* bio-applications.

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Notes and references

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1. T. Kasuga, M. Hiramatsu, A. Hoson, T. Sekino and K. Niihara, *Adv. Mater.*, 1999, **11**, 1307-1311.
2. I. Paramasivam, H. Jha, N. Liu and P. Schmuki, *Small*, 2012, **8**, 3073-3103.
3. X. M. Sun and Y. D. Li, *Chem. Eur. J.*, 2003, **9**, 2229-2238.
4. R. Yoshida, Y. Suzuki and S. Yoshikawa, *Mater. Chem. Phys.*, 2005, **91**, 409-416.
5. A. H. Liu, M. D. Wei, I. Honma and H. S. Zhou, *Anal. Chem.*, 2005, **77**, 8068-8074.
6. S. Mahshid, C. Li, S. S. Mahshid, M. Askari, A. Dolati, L. Yang, S. Luo and Q. Cai, *Analyst*, 2011, **136**, 2322-2329.
7. K. S. Brammer, C. J. Frandsen and S. Jin, *Trends Biotechnol.*, 2012, **30**, 315-322.
8. M. Ray, S. Chatterjee, T. Das, S. Bhattacharyya, P. Ayyub and S. Mazumdar, *Nanotechnology*, 2011, **22**, 415705.
9. A. L. Papa, L. Dumont, D. Vandroux and N. Millot, *Nanotoxicology*, 2013, **7**, 1131-1142.
10. A. L. Papa, L. Maurizi, D. Vandroux, P. Walker and N. Millot, *J. Phys. Chem. C*, 2011, **115**, 19012-19017.
11. C. Mirjolet, A. L. Papa, G. Crehange, O. Raguin, C. Seignez, C. Paul, G. Truc, P. Maingon and N. Millot, *Radiother Oncol*, 2013, **108**, 136-142.
12. S. Barua, J. W. Yoo, P. Kolhar, A. Wakankar, Y. R. Gokarn and S. Mitragotri, *Proc Natl Acad Sci U S A*, 2013, **110**, 3270-3275.
13. M. T. Byrne, J. E. McCarthy, M. Bent, R. Blake, Y. K. Gun'ko, E. Horvath, Z. Konya, A. Kukovecz, I. Kiricsi and J. N. Coleman, *J. Mater. Chem.*, 2007, **17**, 2351-2358.
14. S. K. Mohapatra and M. Misra, *J. Phys. Chem. C*, 2007, **111**, 11506-11510.

15. Y. Gao, X. P. Gao, Y. F. Zhou and D. Y. Yan, *Nanotechnology*, 2008, **19**, 495604.

16. Y. Gao, Y. F. Zhou and D. Y. Yan, *Polymer*, 2009, **50**, 2572-2577.

17. J. Liu, Y. Liu, Z. B. Wu, X. B. Chen, H. Q. Wang and X. L. Weng, *J. Colloid Interface Sci.*, 2012, **386**, 392-397.

18. H. Niu, S. Zhang, X. Zhang and Y. Cai, *ACS Appl Mater Interfaces*, 2010, **2**, 1157-1163.

19. J. Vasquez, Z. Lopez, A. Zuniga, A. Nacher, M. Lira-Cantu, P. Gomez-Romero, M. A. S. Ana, E. Benavente and G. Gonzalez, *Electrochim. Acta*, 2010, **55**, 1373-1379.

20. M. Feng, H. Zhan and L. Miao, *Nanotechnology*, 2010, **21**, 185707.

21. I. Brnardic, M. Huskic, P. Umek, A. Fina and T. H. Grguric, *Phys. Status Solidi A*, 2013, **210**, 2284-2291.

22. P. I. Ponton, J. R. M. d'Almeida, B. A. Marinkovic, S. M. Savic, L. Mancic, N. A. Rey, E. Morgado and F. C. Rizzo, *Appl. Surf. Sci.*, 2014, **301**, 315-322.

23. L. Wang, W. Liu, T. Wang and J. R. Ni, *Chem. Eng. J.*, 2013, **225**, 153-163.

24. G. J. Niu, W. Liu, T. Wang and J. R. Ni, *J. Colloid Interface Sci.*, 2013, **401**, 133-140.

25. Z. Q. Shi, X. P. Gao, D. Y. Song, Y. F. Zhou and D. Y. Yan, *Polymer*, 2007, **48**, 7516-7522.

26. S. Beke, L. Kőrösi, A. Scarpellini, F. Anjum and F. Brandi, *Mater. Sci. Eng., C*, 2013, **33**, 2460-2463.

27. Z. Amoozgar and Y. Yeo, *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, 2012, **4**, 219-233.

28. J. Suh, K.-L. Choy, S. K. Lai, J. S. Suk, B. C. Tang, S. Prabhu and J. Hanes, *Int. J. Nanomed.*, 2007, **2**, 735-741.

29. J. V. Jokerst, T. Lobovkina, R. N. Zare and S. S. Gambhir, *Nanomedicine*, 2011, **6**, 715-728.

30. S. Jo and K. Park, *Biomaterials*, 2000, **21**, 605-616.

31. O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J. P. Behr, *Proc Natl Acad Sci U S A*, 1995, **92**, 7297-7301.

32. R. V. Benjaminsen, M. A. Mattebjerg, J. R. Henriksen, S. M. Moghimi and T. L. Andresen, *Mol. Ther.*, 2013, **21**, 149-157.

33. I. Richard, M. Thibault, G. De Crescenzo, M. D. Buschmann and M. Lavertu, *Biomacromolecules*, 2013, **14**, 1732-1740.

34. T. Kasuga, M. Hiramatsu, A. Hoson, T. Sekino and K. Niihara, *Langmuir*, 1998, **14**, 3160-3163.

35. A. L. Papa, N. Millot, L. Saviot, R. Chassagnon and O. Heintz, *J. Phys. Chem. C*, 2009, **113**, 12682-12689.

36. L. Maurizi, H. Bisht, F. Bouyer and N. Millot, *Langmuir*, 2009, **25**, 8857-8859.

37. F. Ungaro, G. De Rosa, A. Miro and F. Quaglia, *J. Pharm. Biomed. Anal.*, 2003, **31**, 143-149.

38. L. Devillard, D. Vandroux, C. Tissier, L. Dumont, J. Borgeot, L. Rochette and P. Athias, *Mol. Cell. Biochem.*, 2008, **307**, 149-157.

39. A. Grynberg, P. Athias and M. Degois, *In Vitro Cell Dev Biol*, 1986, **22**, 44-50.

40. L. A. Gomez, A. E. Alekseev, L. A. Aleksandrova, P. A. Brady and A. Terzic, *J. Mol. Cell. Cardiol.*, 1997, **29**, 1255-1266.

41. E. Morgado, M. A. S. de Abreu, G. T. Moure, B. A. Marinkovic, P. M. Jardim and A. S. Araujo, *Mater. Res. Bull.*, 2007, **42**, 1748-1760.

42. T. Y. Cao, Y. Yang, Y. Sun, Y. Q. Wu, Y. Gao, W. Feng and F. Y. Li, *Biomaterials*, 2013, **34**, 7127-7134.

43. N. Kohler, G. E. Fryxell and M. Q. Zhang, *J. Am. Chem. Soc.*, 2004, **126**, 7206-7211.

44. K. S. Shin and J. H. Kim, *Bull. Korean Chem. Soc.*, 2011, **32**, 2469-2472.

45. S. S. York, S. E. Boesch, R. A. Wheeler and R. Frech, *Macromolecules*, 2003, **36**, 7348-7351.

Dispersion of titanate nanotubes for nanomedicine: comparison of PEI and PEG nanohybrids

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Titanate nanotubes represent a carrier of interest for drug, imaging agent or DNA delivery because of their (i) morphology, (ii) high specific surface area and (iii) tuneable surface (in terms of easy functionalization capability leading to all possible surface charges). This article focuses on their dispersion and functionalization using PEG (polyethylene glycol) and PEI (polyethyleneimine), two well established standards used for drug delivery and transfection, respectively.