Journal of Virological Methods 168 (2010) 1–5

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Interaction between single wall carbon nanotubes and a human enteric virus

Anna Rita Petrinca[a], Domenica Donia[a], Rosadele Cicchetti[c], Federica Valentini[b], Gabriella Argentin[c], Marilena Carbone[b], Antonio Pietroiusti[d], Andrea Magrini[d], Giuseppe Palleschi[b], Maurizio Divizia[a,*]

[a] University of Tor Vergata, Faculty of Medicine, Dept. Public Health, Hygiene chair, Via Montpellier, 1, 00133 Rome, Italy
[b] University of Tor Vergata, Faculty of Chemistry, Dept. Science and Chemical Technology, Italy
[c] University of Tor Vergata, Faculty of Medicine, Dept. Public Health, Biology Lab, Italy
[d] University of Tor Vergata, Faculty of Medicine, Occupational Medicine, Italy

Article history:
Received 12 October 2009
Received in revised form 30 March 2010
Accepted 30 March 2010
Available online 8 April 2010

Keywords:
Single wall carbon nanotubes
Polyethylenimine
Hepatitis A virus
Real-time reverse transcriptase-polymerase chain reaction

A B S T R A C T

Activated single wall carbon nanotubes have been used for biomedical purposes as carriers for drugs, peptides, proteins and nucleic acids. A large volume of data speaks to their suitability to act as a carrier. The ability of two differently activated SWNTs (with carboxyl groups or with carboxyl groups and polyethylenimine (PEI)) to form a complex with the hepatitis A virus was evaluated. Both types of activations permitted the formation of a virus–SWNT complex. However, their patterns were different. The carboxyl-activated nanotubes had a somewhat low adsorptive capacity that was related inversely to the concentrations of the SWNTs and viruses. Statistical analysis, using the χ^2-test, showed no significant differences between the SWNT–PEI ratios of 1:2.5, 1:1 and 1:0.5. The addiction of PEI improved the adsorption, probably because of the electropositive charge of the molecule. Adsorption was optimal between 100 μg and 10 ng with a SWNTs–PEI weight ratio of 1:0.2 up to an inoculum of 10^5 genome equivalents of hepatitis A virus. Reducing or increasing this weight ratio reduced the adsorptive capacity of the PEI, and this adsorption activity was time and contact-dependent. Thus, SWNTs coated with PEI are able to complex with viruses, and they might be used in the future to transfect non-permissive cell lines.

1. Introduction

Single wall carbon nanotubes (SWNTs) are a stable material with unique chemical, physical and mechanical properties. They are insoluble in any type of solvent, unless they are activated by the non-covalent or covalent addition (Klumpp et al., 2006) of a chemical group to improve biocompatibility and biomolecular properties (Shim et al., 2002). In this condition, they can be used to uptake various molecules such as drugs, proteins and nucleic acids (Kam et al., 2004; Cai et al., 2005) for *in vivo* and *in vitro* delivery. Recently, Brady-Estevez et al. (2008) used single-walled carbon nanotubes to remove viruses and bacteria from water.

Polycationic polymers have been used as functional groups. Among them, polyethylenimine (PEI), which can be of either low or high molecular weight, is one of the most versatile agents (Boussif et al., 1995) for delivery. Its efficiency depends both on the size of the polymer, and on the cytotoxicity associated with the total charge (Fischer et al., 1999). The high molecular weight PEI has a higher transfection efficiency than the low molecular weight version, but it displays dose-dependent cytotoxicity (Nimesh et al., 2006).

The ability of SWNTs, activated with either carboxyl groups and PEI or with carboxyl groups only to withdraw hepatitis A virus (HAV) from a cellular lysate was investigated. In the future, activated SWNTs could be used to transfect non-permissive cell lines or to pick up viruses from different environmental matrices.

The interaction between the SWNTs and the enteric virus (HAV) was demonstrated by direct RT-PCR and quantified with respect to the HA viral control by qRT-PCR.

2. Materials and methods

2.1. Chemicals

SWNTs activated with COOH groups (purity, >90 wt%; diameter, 1–2 nm; length, 5–30 μm; surface area estimated by the BET (Brunauer, Emmett, and Teller) method, 407 m^2/g; and functional COOH-content 2.73 wt%) were purchased from Cheap Tubes (Brattleboro, VT, USA). Branched polyethylenimine (PEI, average

* Corresponding author. Tel.: +39 06 72596119; fax: +39 06 2025285.
E-mail address: divizia@uniroma2.it (M. Divizia).

0166-0934/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2010.03.029

Mw ~ 25,000) was purchased from Sigma–Aldrich (Milan, Italy) and used without any further treatment. Na_2HPO_4, NaH_2PO_4, EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), KCl and ethanol were of analytical grade and were purchased from Sigma–Aldrich (Milan, Italy). Sodium hydroxide pellets were used for pH adjustments. Diluted solutions were prepared just before use. The pH of the solutions was adjusted with either 0.1 M HCl or 0.1 M NaOH.

2.2. Preparation of the carboxylated carbon nanotube PEI nanocomposite (SWNT–PEI)

Following the chemical activation of the COOH groups on the walls of the SWNTs by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), the SWNT–COOH was further activated with PEI, thus yielding SWNT–COOH–PEI nanocomposites (1:2.5% (w/w) is called SWNT–PEI 2.5; 1:1% (w/w) is called SWNT–PEI 1; 1:0.5% (w/w) is called SWNT–PEI 0.5 and 1:0.2% (w/w) is called SWNT–PEI 0.2). Subsequently, these nanocomposites were dispersed in DMEM 1% FCS culture medium (Dulbecco's Modified Eagle's Medium with 1% foetal calf serum) for the molecular tests and in EtOH solution for morphological characterisation by TEM at the concentration of 1 mg/ml using an ultrasonic bath for 30 min at room temperature.

2.3. SWNT dispersion for experiments with HAV

SWNTs activated with 25 kDa PEI or carboxyl groups were dissolved in DMEM (Dulbecco's Modified Eagle's Medium) with 1%FCS (fetal calf serum) (Sager et al., 2007) at a final concentration of 1 mg/ml by sonication in an LBS1 ultrasonic bath (FALC Instruments, Bergamo, Italy) for 2–3 h before each virus-based test. Different amounts of HAV were added to this homogeneous and stable dispersion, and HAV was allowed to conjugate with the SWNT–PEI and SWNT–COOH at room temperature.

2.4. Human hepatitis A virus

HAV (strain HM175 kindly obtained from A. Bosch, Dept. of Microbiology, University of Barcelona) was cultured in a FRhK (fetal rhesus monkey kidney) cell line. Briefly, confluent monolayers growing in Nunc flasks (175 cm^2) (Milan, Italy) were infected with 5 ml of HAV at 1×10^5 gen equiv./ml. After 1 h of adsorption at 37 °C, the inoculum remained and 25 ml of fresh medium was added. At 90–95% of the cytopathic effect (CPE), the cells were frozen and thawed. The suspension was treated with 20% of chloroform to destroy the clumps of virus and was titrated by qRT-PCR.

2.5. Adsorption of viruses onto SWNTs

One hundred microlitres of viral suspension in DMEM 1% FCS at the indicated concentration was mixed in ice with 100 μl of SWNT–PEI or SWNT–COOH at different concentrations. The mixture was washed gently three times with DMEM 1% FCS by centrifugation with a bench Eppendorf centrifuge at 14,500 × *g* for 5 min at room temperature. Each experiment was performed three times.

2.6. Chemical apparatus

SWNT–PEI 0.2 conjugated with HAV and HAV alone (viral control) were characterised by a 100 kV High-Resolution Scanning Transmission Electron Microscope (HRTEM) using a TEM Philips (CM120, with LaB6 as filament). For the TEM investigation, the SWNT–PEI 0.2-HAV nanocomposite was dispersed previously in ethanol (1 mg/5 ml) using an ultrasonic bath for 1 h at room temperature. Following the same procedure, an HAV solution was prepared in ethanol and used as a control. A perforated carbon Formvar film supported by copper grids (3 mm) was dipped into the SWNT–PEI 0.2-HAV and HAV control for a few seconds. The solvent was evaporated subsequently with an IR lamp.

2.7. Virus detection by reverse transcriptase-polymerase chain reaction (RT-PCR)

Viral RNA was extracted from pellets using the Trizol-LS reagent (Invitrogen, Milan, Italy). The final RNA was collected in 20 μl of sterile RNA-DNase free water (Invitrogen, Milan, Italy); 10 μl was reverse transcribed immediately, and the total cDNA was amplified using specific primers designed from the VP1–VP2 region of the viral genome by OligoExplorer 1.2 software: HAV-F: 5′-TGA ACA GGT ATA CAA AGT CAG C-3′, position 2017–2038 of the human hepatitis A strain (accession number M14707); HAV-R: 5′-ATA CCA ACT TGG GGA TAT GGA AC-3′, position 2281–2259. Primer HAV-R at a concentration of 1 μM was used in an RT reaction in 30 μl (final volume) containing 4 U of AMV Reverse Transcriptase (Promega, Milan, Italy), 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega, Milan, Italy), each deoxynucleoside triphosphate at a concentration of 0.2 mM and 10 μl of denatured (5 min at 95 °C) RNA samples. The reaction mix was incubated for 30 min at 42 °C. The RT product was processed by PCR using 2 U of GoTaq Flexi DNA Polymerase (Promega, Milan, Italy) in a 100 μl mixture, supplemented with each primer at a concentration of 0.5 μM, each deoxynucleoside triphosphate at a concentration of 0.2 mM and $MgCl_2$ at a concentration of 2 mM. The PCR program included a 3 min denaturation step at 95 °C and 45 cycles of amplification for 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, followed by a final elongation step of 5 min at 72 °C. The PCR product of 265 bp was loaded on a 2% agarose gel containing 0.5 μg/ml of ethidium bromide and then photographed under UV light.

2.8. Virus titration by real-time RT-PCR (qRT-PCR)

The HAV titre (2.7×10^4 copies/μl) was obtained by qRT-PCR using SYBR Green I chemistry and following an in house protocol. Armored RNA Hepatitis A Virus (Ambion Diagnostic, USA) was used as a standard to generate a calibration curve from 10-fold dilutions of the initial standard (Donia et al., 2006). Once HAV was quantified, extracted and purified viral RNA was used to generate a standard curve to determine viral adsorption in SWNT tests (the second standard curve). The primers were the same as those used for RT-PCR and were reported previously. Syber Green I real-time one step RT-PCR was carried out in a 25 μl reaction volume with a BioRad iCycler. Five microlitres of extracted RNA were assayed in three replicates containing 20 μl of the master mix supplied with the kit (QuantiTect Syber Green I RT-PCR kit, Qiagen, Hilden, Germany), added to 0.4 μM each of the forward and reverse primers. After reverse transcription at 50 °C for 30 min, Taq polymerase was activated at 95 °C. Amplification was undertaken for 45 cycles consisting of a denaturation step at 94 °C for 15 s, annealing at 55 °C for 30 s and an extension step at 72 °C for 30 s. The threshold cycle values generated by the iQ analysis software were used to calculate a linear regression line by plotting (on a graph) the logarithm of the starting copy number, expressed as increasing fluorescence values from the standard dilution amplification. The slope of the standard curves determined the PCR efficiency (EFF). The reproducibility of the qRT-PCR was indicated by correlation coefficients (CC) with values ranging between 0 and 1 (Donia et al., 2005). A melting curve protocol was used to determine the specificity of amplification. The presence of a single melting temperature peak indicated a specific product (79 ± 0.5 °C).