Materials Science and Engineering C 39 (2014) 359–370

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Carbon nanoparticles for gene transfection in eukaryotic cell lines

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ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form 18 February 2014
Accepted 4 March 2014
Available online 12 March 2014

Keywords:
Gene transfection
Cell viability
Metabolomic test
Carbon nanomaterial

ABSTRACT

For the first time, oxygen terminated cellulose carbon nanoparticles (CCN) was synthesised and applied in gene transfection of pIRES plasmid. The CCN was prepared from catalytic of polyaniline by chemical vapour deposition techniques. This plasmid contains one gene that encodes the green fluorescent protein (GFP) in eukaryotic cells, making them fluorescent. This new nanomaterial and pIRES plasmid formed π-stacking when dispersed in water by magnetic stirring. The frequencies shift in zeta potential confirmed the plasmid strongly connects to the nanomaterial. In vitro tests found that this conjugation was phagocytised by NG97, NIH-3T3 and A549 cell lines making them fluorescent, which was visualised by fluorescent microscopy. Before the transfection test, we studied CCN in cell viability. Both MTT and Neutral Red uptake tests were carried out using NG97, NIH-3T3 and A549 cell lines. Further, we use metabolomics to verify if small amounts of nanomaterial would be enough to cause some cellular damage in NG97 cells. We showed two mechanisms of action by CCN–DNA complex, producing an exogenous protein by the transfected cell and metabolomic changes that contributed by better understanding of glioblastoma, being the major finding of this work. Our results suggested that this nanomaterial has great potential as a gene carrier agent in non-viral based therapy, with low cytotoxicity, good transfection efficiency, and low cell damage in small amounts of nanomaterials in metabolomic tests.

1. Introduction

One of the greatest advantages of nanotechnology is the ability to translocate across cell membranes, expressing functions for regulation or repair [1]. Gene delivery is an emerging tool to express or assign functions to a nucleic acid sequence to achieve on/off regulation of the endogenous genes or repair missing/defective genes for therapeutic intervention [2–4]. Methods of gene transfection of cells and tissues range from bacterial to mammalian. Effective virus-based systems carry risks, and thus, efficient synthetic systems that are non-toxic need to be found. Non-viral vectors such as cationic polymers, lipids, and peptides have several advantages such as low immunogenicity, target-cell specify, and relative safety profiles. However, their low transfection efficiency compared to virus systems, combined with their high toxicity, is a significant obstacle for their application [2,5]. Therefore, extensive effort has been made to develop new materials for highly efficient gene transfection with low cytotoxicity. In recent years, carbon-based nanomaterials have attracted attention in various fields to explore their potential applications. Further, carbon-based materials are considered attractive candidates for biomedical applications due to their outstanding properties such as high surface area, lightweight, exceptional mechanical elasticity, large carrier mobility, biocompatibility, and facility for functionalisation [6,7].

Graphene oxide and carbon nanotubes are currently being used in engineered tissues [6,8], implants [6,9], diagnostic tools and chips [6,10,11], biological imaging [6,12], drug delivery carriers [6,13], and antibacterial materials [6,14]. However, one problem that limits their use in nanobiology and nanomedicine is that their pristine forms are largely not dispersible in water. Those materials precipitate in such solvents because of agglomeration. Usually, the surface material functionalisation is the key to allow effective application. In addition, several studies suggest low biocompatibility of carbon nanotubes (CNT), while others give the opposite conclusion [15–18]. Other studies found an obvious preference for cell growth on a CNT surface. Several studies highlighted the carbon based materials interferes with cytotoxicity dyes, commonly used to study cell integrity, viability, and proliferation [19–24]. The toxicity of CNT is attributed to their physicochemical properties, including structure, length and aspect ratio, surface area, degree of aggregation, extent of oxidation, surface topology, bound functional group(s), manufacturing method, concentration, and dose offered to cells or organisms [1,25–28]. CNT can cause toxicity through membrane damage, DNA damage, oxidative stress, changes in mitochondrial activities,

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http://dx.doi.org/10.1016/j.msec.2014.03.016
0928-4931/

altering intracellular metabolic routes, and protein synthesis [1,29–34]. The most common mechanisms of CNT cytotoxicity also involve apoptosis and necrosis [1,17,23,35]. However, CNT cytotoxicity is controversial, with a large number of studies reporting altered toxic responses to CNT both in vitro and in vivo [1,16,1,29,36–38]. More studies are desirable to develop best practices for the actual evaluation of toxicity. That is why these materials are attractive as biomaterials [39–43].

For instance, DNA can bind to single-walled carbon nanotube (SWCNT), forming tight helices around them [44], or can form noncovalent conjugates with CNT [1,45]. CNT wrapped with flavin mononucleotide and DNA enhances dispersion of these nanotubes [1, 46,47]. DNA–functionalised CNT can be used as biological transporters and as biosensors [1,48]. DNA-encased multi-walled carbon nanotubes (MWCNT) were more effective than plain MWCNT against malignant tissues when tested in vivo for their thermal ablation capability [1,49]. DNA–CNT could penetrate lymphocytes instantly with a needle-like mechanism, thus reducing cytotoxic effects [1,50]. CNT was found to be similar to cell-penetrating proteins, because they can penetrate cells without endocytosis [51]; however, the internalisation of nanomaterials depends on the functionalisation process [1]. Besides, the interactions between proteins and CNT could play a key role in the biological effects of CNT [52,53]. A π–π stacking occurs between CNT and aromatic residues (Trp, Phe, Tyr) of proteins, enhancing their absorptivity and biocompatibility, which reduces their toxicity compared to pristine CNT [1,54–56]. CNT–protein nanoconjugates are useful in biosensor fabrication [57], drug delivery [58], and cancer therapy [1, 56,59].

The primary objective of our study was to evaluate the non-toxicity and efficacy of CCN for gene delivery. For this, we used NG97, NIH-3T3 and A549 cells lines. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) and Neutral red (NR) tests showed their non-cytotoxicity dose level. In addition, we inserted CCN conjugated with pIRES plasmid into the NG97, NIH-3T3 and A549 cells. The complex plasmid–nanomaterial was internalised by the cell with endocytosis process, which instantly transferred to these cell lines the plasmid fluorescence, making them fluorescent, which was observed in a fluorescence microscope. Besides, we reported for the first time, using metabolomic assay that a low concentration of CCN induced the production of N-stearoyl-alanine and N-oleyl-alanine in cells for type III astrocytoma.

2. Experimental

2.1. Synthesis of cellulose carbon nanoparticles (CCN)

Prior to the deposition, 40 mm × 10 mm dried bamboo substrates were immersed in 1 ml polyethylene diluted in 2 ml of dimethylformamide and then dried at room temperature for 2 h. We inserted the bamboo substrate into hot-filament chemical vapour deposition reactor, which was fed with a solution of 1:1 acetone and camphor vapour dragged into the reactor by 20 sccm of hydrogen flux. We obtained CCN using 100 sccm of nitrogen atmosphere for a half hour. We maintained the pressure inside the reactor at 20 Torr, the temperature of the tungsten filament at 2000 °C and the bamboo substrate temperature at 450 °C (measured at the backside). After growth, we milled the CCN using a ball mill for reduction of agglomerates and then sonicated in an aqueous solution.

2.2. Characterisation of carbon nanoparticles

We characterised the CCN by high-resolution scanning electron microscopy (HR-SEM), as well as Raman and Fourier transform infrared (FTIR) spectroscopies. The JEOL 6330 microscope operated at 20 kV and coupled with an energy dispersive X-ray spectrometer (EDS) operating with a Si (Li) detector with an energy resolution of 126 eV characterised the morphological and chemical analysis, respectively. For the particles size distribution analyses, we performed a spraying the colloidal solution of CCN onto titanium substrate. We heated and rotated the substrate in whole the spraying process. Raman spectra were recorded at ambient temperature using a Renishaw microprobe system, employing an argon laser for excitation ($\lambda = 514.5$ nm) with a laser power of approximately 6 mW. Fourier transform infrared spectroscopy (FTIR: Spotlight 400 — Perkin-Elmer) was used to identify the oxygen content groups on graphene. We conducted all measurements at room temperature. We analysed Zeta potential and particle size applied dynamic light scattering (DLS, Zetasizer, Malvern — UK). All samples were diluted at 150 μg ml^{-1} in KCl 1 mm solution and were de-agglomerated in two different ways, i) conventional sonication for 30 min and ii) ultra-power irradiation at 200 W for 30 min. Follows, we placed the CCN on a polystyrene zeta cuvette. We performed the measurements during 2 h after re-suspended.

2.3. Biological in vitro

2.3.1. Maintenance of NG97, NIH-3T3 and A549 cell culture

The NIH-3T3 (fibroblast embryonary of *Mus musculus*, purchased from ATCC® CRL-1658™), A549 (adenocarcinomic human alveolar basal epithelial cells, purchased from ATCC® CCL-185™), and NG97 (astrocytoma type III, developed and donated by Prof. Dra. Eliana Verinaud [60]) were used to investigate the cytotoxicity and gene transfection of plasmid–nanomaterial substrates. A549 cells were chosen because they are widely used as an in vitro model for a type II pulmonary epithelial cell model for drug metabolism and as a transfection host [6,61]. We chose the NIH-3T3 cells because they are widely used as an in vitro model for a tissue model. Therefore, if the material tested is cytotoxicity on this cell, their use in therapy is discouraged. NG97 cell line was derived from a human astrocytoma grade III, which started to develop and express important phenotypical characteristics of an astrocytoma grade IV after injection in the flank of nude mice. Astrocytomas are extremely aggressive malignancies of the Central Nervous System (CNS) and account for 46% of all primary malignant brain tumours. Progression to worse prognosis occurs in 85% of the cases possibly due to changes in cell tumour microenvironment and through biological pathways that are still unclear [60]. Therefore, if plasmid can be transfected into this cell, in the future, genes could be transfected to fight these diseases, and if plasmid–nanomaterial was not cytotoxic for NIH-3T3, this complex could be used in transfection assays. NG97, NIH-3T3, and A549 cells were grown in plastic flasks (25 cm^2) with RPMI 1640 medium (Cultilab, Brazil), supplemented with 10% inactivated foetal bovine serum (Crypion, Brazil) and 1% antibiotic/antimycotic solution (Cultilab, Brazil). The cultures were incubated at 37 °C in an atmosphere containing 5% CO_2. Medium was changed every 48 h, and when the culture reached confluence, the subculture was treated with trypsin (Cultilab, Brazil) [62,63].

2.3.2. MTT and NR assays

Before testing, we dispersed 1 mg ml^{-1} of CCN in culture medium. We plated the NG97, NIH-3T3 and A549 cells separately in 96-well plates. We used 1×10^5 cells ml^{-1} to 1×10^7 cells ml^{-1} per well, respectively. After this, we incubated at 37 °C, in a humidified incubator with 5% CO_2, for 24 h. Then, the medium was replaced, and the test repeated with different CCN concentrations in the range of 3.9 to 500 μm ml^{-1}, which was added to the wells in triplicate for each concentration. We used DL50 to define the CCN cytotoxicity (50% of the cells died).

2.3.2.1. MTT reduction assay. After 24 h incubation, we removed the medium containing CCN and washed 3 times with 0.1 ml of PBS Buffer (137 mM NaCl, 10 mM phosphate, KCl 2.7 mM, and a pH of 7.4) the wells. Next, 0.2 ml of RPMI 1640 medium (without FBS and antibiotic) containing the dye MTT (0.5 mg ml^{-1}) was added. After incubation for 3 h at 37 °C, we removed the medium with dye and carefully added 0.2 ml of ethanol to solubilize the blue formazan (yielded from