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Characterization of different carbon nanotubes for the development of a mucoadhesive drug delivery system for intravesical treatment of bladder cancer

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ABSTRACT

In order to increase the effectiveness of therapeutics for bladder carcinoma (BCa) treatment, alternative strategies for intravesical applications are needed. The use of carbon nanotubes (CNTs) as basis for a multifunctional drug transporter is a promising possibility to combine traditional chemotherapeutics with innovative therapeutic agents such as antisense oligodeoxynucleotides or small interfering RNA. In the current study four CNT types varying in length and diameter (CNT-1, CNT-2, CNT-3, CNT-4) were synthesized and then characterized with different spectroscopic techniques. Compared to the pristine CNT-1 and CNT-3, the shortened CNT-2 and CNT-4 exhibited more defects and lower aspect ratios. To analyze their mucoadhesive properties, CNTs were exposed to mouse bladders *ex vivo* by using Franz diffusion cells. All four tested CNT types were able to adhere to the urothelium with a mean covering area of 5–10%. *In vitro* studies on UM-UC-3 and EJ28 BCa cells were conducted to evaluate the toxic potential of these CNTs. Viability and cytotoxicity assays revealed that the shortened CNT-2 and CNT-4 induced stronger inhibitory effects on BCa cells than CNT-1 and CNT-3. In conclusion, CNT-1 and CNT-3 showed the most promising properties for further optimization of a multifunctional drug transporter.

1. Introduction

Bladder carcinoma (BCa) is the most common malignancy of the urinary tract. In Europe, BCa had a rate of 151,189 new cases and 52,358 tumor-related deaths in 2012 (http://eu-cancer.iarc.fr/EUCAN/Cancer.aspx?Cancer=32, 02-11-2014). At first diagnosis, 75–85% of the BCa are confined to the mucosa (tumor stage pTa and carcinoma *in situ*) or submucosa of the urinary bladder (tumor stage pT1). Tumors of these stages are grouped as non-muscle invasive bladder cancer (NMIBC) (Anastasiadis and de Reijke, 2012). Transurethral resection of the bladder tumor (TUR-BT) is the initial treatment of choice for NMIBC and is performed to remove all visible lesions (Anastasiadis and de Reijke, 2012). After TUR-BT, patients receive an adjuvant intravesical chemotherapy or immunotherapy to lower the recurrence rate and to prevent or delay progression to muscle-invasive disease (Babjuk et al., 2013). Recurrence rates in patients with NMIBC range from 31% to 78% within 5 years from diagnosis in the low-risk and high-risk subgroups, respectively (Babjuk et al., 2013).

Abbreviations: BCa, bladder carcinoma; CNT, carbon nanotube; CVD, chemical vapor deposition; DMEM, Dulbecco's Modified Eagle Medium; DOX, doxorubicin; H&E, hematoxylin–eosin; MEM, Minimum Essential Medium; MTC, magnetically targeted carrier; MW-CNT, multi-walled carbon nanotube; NMIBC, non-muscle invasive bladder cancer; PBS, phosphate buffered saline; RT, room temperature; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TGA, thermogravimetric analysis; TUR-BT, transurethral resection of the bladder tumor.

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These high rates of recurrence and a significant risk of progression in high-grade tumors require additional and improved therapy strategies which might be combined with traditional intravesically applicable agents. Because of its structure as a hollow organ the urinary bladder is suitable for a local drug administration, where the therapeutics are simply instilled as solution through a catheter. However, intravesically administered therapeutics are rapidly diluted and lose their efficiency due to the continuous production of urine. The use of a multifunctional transporter for conventional chemotherapeutics as well as innovative therapeutic agents, such as nucleic acid-based expression inhibitors of tumor-associated genes, might represent a promising strategy to circumvent these obstacles. To increase the therapeutic effectiveness, the drug transporter should stick to the urothelium – the epithelial layer of the bladder – and release the therapeutics directly at the BCa cells. Furthermore, the multifunctional transporter could meet the requirements of high local doses and sufficient dwell time of different therapeutics.

Numerous strategies for intravesical drug delivery systems have been reported, whereby nanocarriers represent the most widely used technology (Barthelmes et al., 2011; Chang et al., 2009; Hsu et al., 2013; Leakakos et al., 2003). Carbon nanotubes (CNTs) might be potentially useful as multifunctional drug delivery vehicles (Arlt et al., 2010; Eatemadi et al., 2014; Fadel and Fahmy, 2014; Hampel et al., 2008; Mendes et al., 2013). CNTs are hollow graphitic fibers and can be functionalized on the surface due to the sp^2 hybridization of the carbon atoms. The introduction of functional groups on the surface can enhance the solubility and biocompatibility of CNTs (Liu et al., 2009; Tasis et al., 2006). Small variations in CNT morphology and physicochemical features could also modulate their toxicity (Muller et al., 2008; Ringel et al., 2014). For example, surface chemistry and aspect ratio – the ratio between length and diameter of CNTs – can influence the viability of cells (Bottini et al., 2006; Liu et al., 2009). Magrez et al. exposed different cell types to carbon-based nanomaterials with different aspect ratios to explore the dependence of toxicity on structure and size of such materials. They observed that increased cell death and inhibition of cell proliferation was related to a lower aspect ratio (Magrez et al., 2006).

CNTs can serve as drug delivery containers due to their hollow structure which allows the encapsulation of drugs. Previously, we have shown that CNTs can be loaded with the conventional chemotherapeutic carboplatin which was continuously released and could exert its anticancer activity *in vitro* (Arlt et al., 2010; Hampel et al., 2008). Furthermore, CNTs can function as carriers for nucleic acid-based expression inhibitors such as antisense oligodeoxynucleotides or small interfering RNA as described in several studies (Bates and Kostarelos, 2013; Liu et al., 2009; Mendes et al., 2013; Nandy et al., 2012; Nicolas et al., 2013; Wong et al., 2013). Nevertheless, for a successful utilization in BCa treatment, their adhesion to the urothelium still has to be proven. To study the mucoadhesion of such drug transporters, a variety of methods has been developed (Barthelmes et al., 2011; Leakakos et al., 2003). In this regard, the use of Franz diffusion cells could be very helpful as shown by Chang et al. in permeability studies on porcine urinary bladders (Chang et al., 2009).

The aim of this study was the characterization of different synthesized multi-walled CNTs (MW-CNTs) with varying aspect ratios to identify the best CNT type which could serve as multifunctional transporter. Different spectroscopic techniques have been applied to characterize the morphological and physical properties of the different MW-CNT types. Potential inhibitory effects on BCa cells were assessed by *in vitro* measurements of the cellular viability, cytotoxicity and induction of apoptosis. *Ex vivo* mucoadhesion studies on explanted mouse urinary bladders were conducted by using Franz diffusion cells to reveal the quality and quantity of mucoadhesion of the different CNT types to the urothelium. The present study serves as the starting point for the development of a CNT-based drug delivery system to be locally applied into the bladder for BCa treatment.

2. Materials and methods

2.1. Synthesis and shortening of CNTs

The CNTs were synthesized by the fixed-bed chemical vapor deposition (CVD) method as reported by Ritschel et al. (2007). Two different sizes of iron catalyst particles were used to produce CNTs with different diameters (Fig. 1). Smaller catalyst particles were used to grow CNTs with a mean diameter of 11 nm (CNT-1) and larger catalyst particles produced CNTs with a mean diameter of 18 nm (CNT-3). The catalyst particles were removed by washing with 32.5% nitric acid (VWR, Darmstadt, Germany), which also lead to an oxidation at the ends of the CNTs.

For CNT shortening 50 mg of CNT-1 or CNT-3, 20 g Zirconia (ZrO_2) balls (Ø = 0.5 mm; Fritsch, Idar-Oberstein, Germany) and 5 ml ethanol (denatured with 1% methylethylketone; Berkel AHK, Berlin, Germany) were put into an agate beaker of a ball mill (Pulverisette 7 premium line, Fritsch, Idar-Oberstein, Germany). The mixture was milled at 700 rpm for 15 min. Thereafter, the grinding was paused for 45 min to cool down the mixture. This procedure was repeated five times. Subsequently, the shortened CNTs were dispersed in ethanol to separate them from the ZrO_2 balls. After decantation, the CNTs were filtrated through a teflon filter with 0.45 μm pore size (Satorius Stedim Biotech, Göttingen, Germany). This process was rerun until no more CNTs were separated. Finally, the CNTs were dried overnight at 108 °C. CNT-1 were shortened to CNT-2 and CNT-3 were shortened to CNT-4 (Fig. 1).

The CNTs were individualized by sonication in 20 ml isopropyl alcohol (Promochem, Wesel, Germany) in a sonication bath (Bandelin, Berlin, Germany) for 30 min. The resulting gray and transparent dispersion was dropped onto a Cu-formvar grid for transmission electron microscopy (TEM; Plano, Wetzlar, Germany) as well as scanning electron microscopy (SEM; NanoSEM, FEI Company, Hillsboro, OR, USA) using 15 kV acceleration voltage. The length of individual CNTs was evaluated using the iTEM software (Version 5.2, Olympus Soft Imaging Solutions; Münster, Germany). To estimate the sp^2 to sp^3 ratio of carbon – as measure of the defect rate – Raman spectroscopy measurements were performed using a Raman–Fourier-Transform-Spectrometer DXR SmartRaman (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 532 nm and laser power of 8 mW. The resolution of the spectrometer is 1 cm^{-1}. Thermogravimetric analysis (TGA) was applied to study the thermal stability of CNTs and performed in a SDT Q600 (TA Instruments, Eschborn, Germany) with a heating rate of 10 K/min at 100 ml/min synthetic air flow.

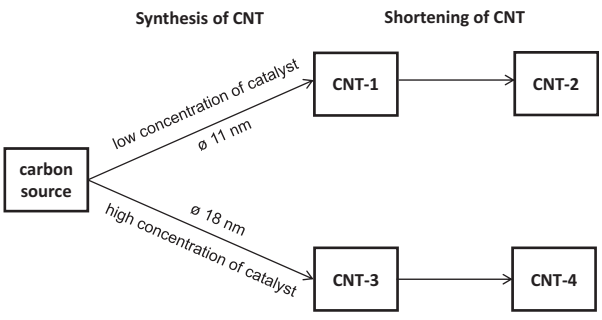

Fig. 1. Flow chart of synthesis and shortening of the different CNT types.