RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

RSC Advances

RSCPublishing

COMMUNICATION

Gene delivery to Nile tilapia spermatogonial stem cells using carboxi-functionalized multiwall carbon nanotubes

Cite this: DOI: 10.1039/x0xx00000x

Received 00th May 2014,
Accepted 00th January 2014

DOI: 10.1039/x0xx00000x

www.rsc.org/

F.M.P. Tonelli,[a] S.M.S. N. Lacerda,[b] M.A. Silva, [b] E.S. Ávila, [c] L.O. Ladeira,[c] L.R. França, [b] and R.R. Resende[a]

Carboxi-functionalized multiwall carbon nanotubes (fMWCNTs), when complexed with DNA, can promote gene delivery to Nile tilapia spermatogonial stem cells with higher transfection efficiency than cationic lipids or electroporation, causing also less cell death.

Studies on gene transfer to fish cells have been made since the 80s[1] using different techniques such as electroporation[2], proteins conjugated to poliamine[3], cationic lipids[4], microinjection of newly fertilized egg[5], among others. However, despite significant and recent progress, frequently, these methods have shown limited success due to technical difficulties, resulting in low efficient rate of transfection and consequently high cost. With the aim of finding different gene delivery systems to deliver genes or plasmids to cells, alternative gene carriers have been developed[6-8]. Among them, are the functionalized carbon nanotubes whose potential as gene transfer vectors has been explored and generated promising results[9-11]. However, this important ability of carbon nanotubes has not yet been evaluated in fish cells.

When it comes to fish cells, spermatogonial stem cells (SSCs) are noteworthy. Because of its unique ability to transmit genetic information to offspring through the male gamete, the genetic modification of SSC represents an excellent alternative strategy to produce transgenic animals, particularly if we consider that usually a single spermatogonial stem cell gives rise to several hundred sperm[12]. Fish production through transgenic techniques offers many potential economic advantages for commercial aquaculture production, including introduction of new or novel traits and increased response to selection for faster growth.

Here, we report for the first time the use of carboxi-functionalized multiwall carbon nanotubes (fMWCNTs) to transfect Nile tilapia (*Oreochromis niloticus*) SSCs, achieving higher transcription level of transgene and less cell death when compared to cationic lipids (Lipofectamine 2000® - Life Technologies) and electroporation, conventional methods to perform the gene delivery.

MWCNTs (Fig. 1a and 2a) were produced by the chemical vapor deposition method (CVD) using cobalt and iron as catalysts, and ethylene as the carbon source. Subsequently, carbon nanotubes underwent acid treatment to remove impurities, catalyst and amorphous materials. They were functionalized through oxidation in nitric/sulfuric acid[13] for 15 minutes, using microwaves. The fMWCNTs were then washed in neutral pH, and dried. Carboxyl group functionalization of MWCNTs was confirmed by FT-NIR (Fourier Transform Near-Infrared spectroscopy). The CNTs had an average length of 200 nm, and an average outer diameter of 25 nm; through thermal analysis of fMWCNTs it was determined the weight loss related to carboxylic function: 6,36%.

The plasmid DNA (6121 pb – 20 nM) containing the endogenous *O. niloticus* β-actin promoter, and the gene of AmCyan1 fluorescent protein was conjugated to the fMWCNT (Fig. 1b and 2b) (0.25 mg/mL) through ultrasonic bath (25 kHz, 100 W for 30 minutes). The attachment was confirmed through resonant Raman spectroscopy assisted by optical microscopy (Fig. 1c). The complex fMWCNT-DNA formation induced a downshift (at ~ 8cm^{-1}) of the tangential G band (localized at ~ 1580 cm^{-1}). This behavior is essentially attributed to the charge transfer between the oxygen atoms on the surface of the fMWCNTs and the electron donating groups present in the DNA, such as amine groups[14].

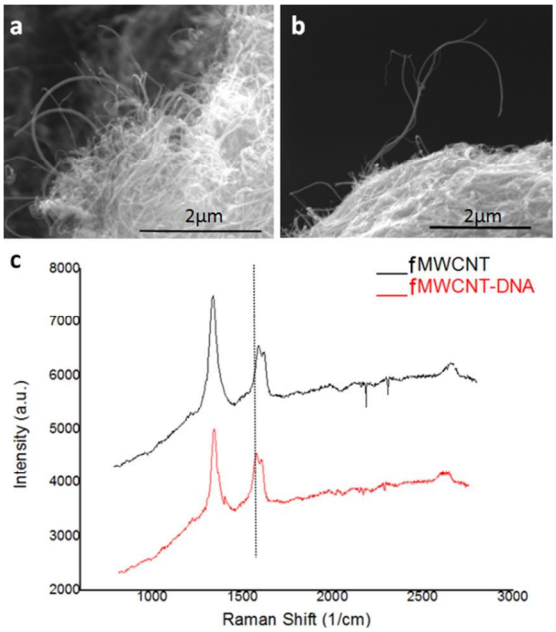

Fig.1-a. Field Emission Gun Scanning Electron Microscopy (FEG-SEM) image of MWCNTs. **b.** FEG-SEM image of fMWCNTs. **c.** Raman spectra obtained for nanotubes (fMWCNTs), and the complex (fMWCNT-DNA). The G band downshift induced by the complex formation is highlighted.

The complex fMWCNT-DNA was added to primary culture of Nile tilapia SSCs (10^6cell/dish) obtained by density gradient centrifugation and differential plating[15]. The gene delivery was also performed by Lipofectamine 2000® (Fig. 2c) and electroporation (225 V e 50 µF). Cells were then incubated for 24 hours at 28ºC and 5% CO_2. After this period, internalization of plasmid DNA into cells and induction of transgene expression were accessed by fluorescence microscopy (Fig.3), and cells viability through flow cytometry (Fig.4) using the Annexin-V-FLUOS staining kit (Roche).

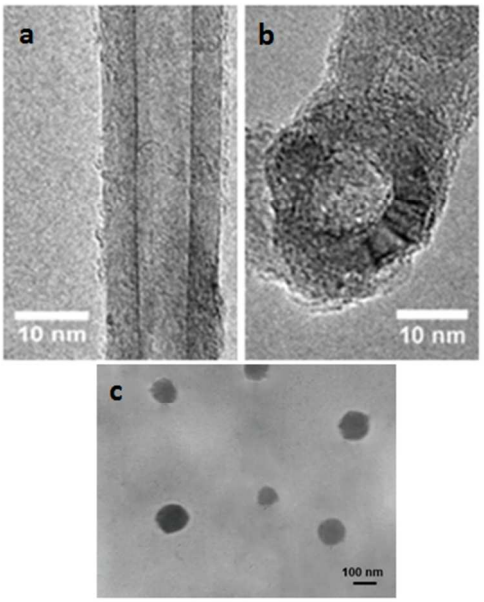

Fig.2- a. Transmission Electron Microscopy (TEM) image of MWCNT. **b.** fMWCNT. **c.** Lipofectamine 2000®.

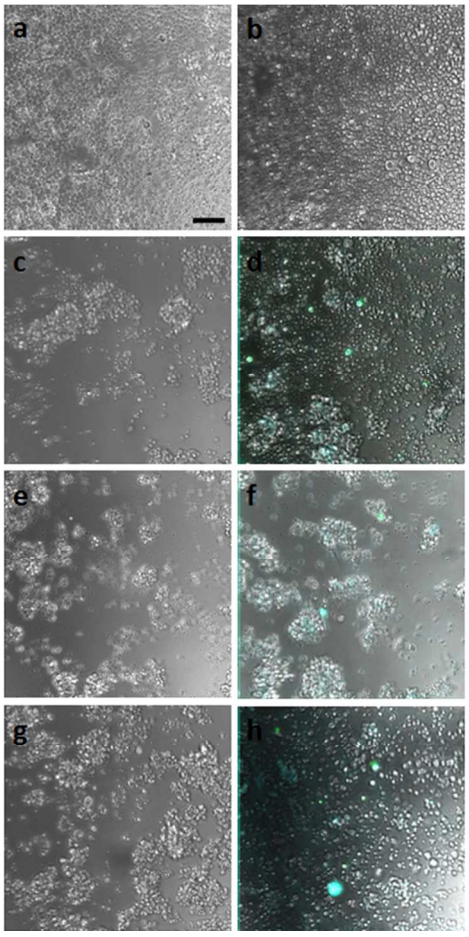

Fig.3-Fluorescence microscopy images of SSCs culture; **a.** control; **b.** DNA 20nM and no vehicle; **c.** fMWCNTs; **d.** fMWCNTs-DNA 20nM; **e.** electroporation; **f.** electroporation + DNA 20nM; **g.** Lipofectamine 2000®; **h.** Lipofectamine 2000®+ DNA 20nM. Bar=50µm.

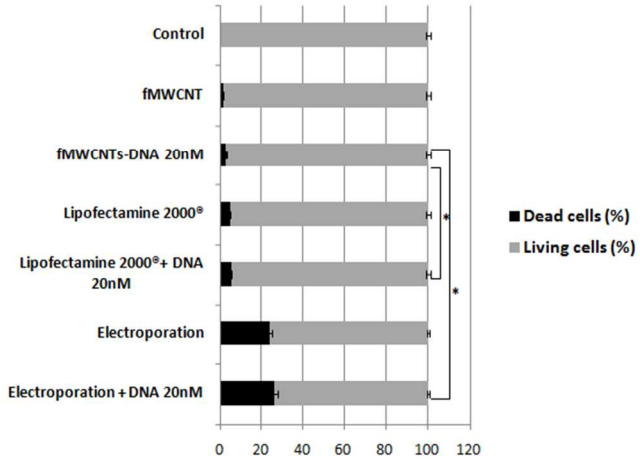

Fig. 4- Dead and living cells percentage obtained through flow cytometry for the different methods of gene delivery tested (asterisk, $p<0.05$, *t* test).

The cyan fluorescence in SSCs was observed for all techniques/vehicles of gene delivery investigated (Fig.3d, f and h),

and in the absence of plasmid DNA no fluorescence was detected for these techniques (Fig.3c, e and g). As expected, when only the DNA (20nM), without any delivery vehicle, was added to SSCs culture (Fig.3b) it did not induced AmCyan1 expression, showing therefore no passive incorporation of plasmids by the fish cells.

Cell death rate observed in transfections using fMWCNTs was significantly lower compared to those in which Lipofectamine or electroporation were applied to gene delivery. These results indicate that fMWCNTs have low cytotoxic effect on Nile tilapia SSCs at the concentration used. Similarly to results obtained for zebrafish neurons[16], higher cytotoxicity was induced by electroporation.

SSCs RNA were extracted with TRIzol® reagent (Life) and underwent RT-PCR using oligodT primer. To access transcription level of AmCyan1 fluorescent protein, q-PCR (Fig. 5) was performed using the cDNA generated, and primers: for the housekeeping gene β-actin (FWD-CGGTATGGAGTCTTGTGGTATC; REV-AGCACAGTGTTGGCGTATAA) and for the AmCyan1 gene (FWD-TTCGAGAAGATGACCGTGTG; REV-AGGTGTGGAACTGGCATCTGTA).

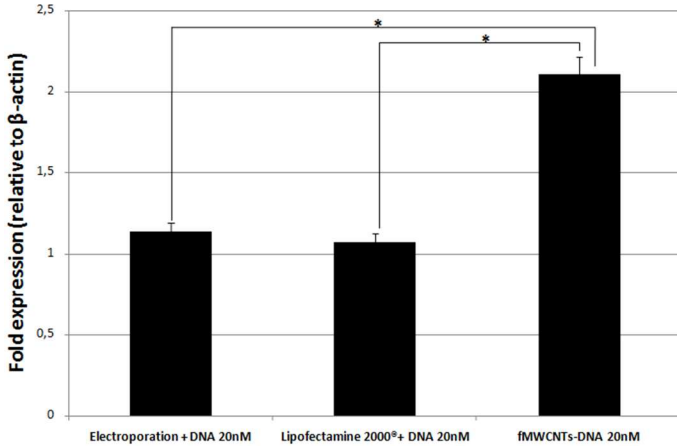

Fig. 5- Dosage of transgene's expression by qPCR. The values correspond to expression of AmCyan1 mRNA in relation to β-actin's in Nile SSCs using different methods of transfection(asterisk, $p<0.05$, *t* test).

Our experimental data revealed that the transcription level of AmCyan1 gene is higher when the gene delivery is performed using the fMWCNT. This expression was 2.1 (± 0.1) fold higher than the β-actin and also significantly superior to the expression achieved by electroporation or Lipofectamine. In zebrafish fibroblasts, low efficiency of transgene expression was previously observed using Lipofectamine. This limitation could be due to intracellular barriers as, for example, poor endocytosis [17].

Conclusions

In conclusion, we reported for the first time success in the use of carbon nanotubes to perform the gene delivery to a fish cell. The fMWCNTs were able to transfect the Nile tilapia SSCs inducing low cytotoxicity. Cell death percentage obtained was lower than those observed for electroporation and cationic lipids, which are commonly used for gene transfection. Moreover, fMWCNTs were able to deliver plasmid DNA more efficiently than the other methods tested. The expression level of cyan fluorescent protein gene related to β-actin, was significantly higher when exposing SSCs to the complex fMWCNT-DNA, instead of using Lipofectamine 2000® or electroporation.

Notes and references

[a]Cell Signaling and Nanobiotechnology Laboratory, Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, Brazil.
[b]Cell Biology Laboratory, Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, Brazil.
[c]Nanomaterials Laboratory, Department of Physics and Center of Microscopy, Federal University of Minas Gerais, Belo Horizonte, Brazil.
† SeeDOI: 10.1039/c000000x/

1 G. Brem, B. Brenig, G. Hörstgen-Schwark and E. Winnacker, *Aquaculture,* 1998, **68**, 209—219.
2 K. Schuster and A. Ghysen, *Cold Spring Harb Protoc.,* 2013, **12**, 1169-1171.
3 Y. Yan, N. Hong, T. Chen, M. Li, T. Wang, G. Guan, Y. Qiao, S. Chen, M. Schartl, C.M. Li and Y. Hong, *PLoS ONE,* 2013, **8**, e59400.
4 A. M. Sandbichler, T. Aschberger and B. Pelster, *Biores Open Access,* 2013, **2**, 20-27.
5 L. Zhang, C. Sun, X. Ye, S. Zou, M. Lu, Z. Liu and Y. Tian, *Fish Physiol Biochem.,* 2014, **40**, 221-233.
6 K. C. F. Leung, C. P. Chak, S. F. Lee, J. M. Y. Lai, X. M. Zhu, Y. X. J. Wang, K. W. Y. Sham and C. H. K. Cheng, *Chem. Commun.*, 2013, **49**, 549-551.
7 Y. Tao, E. Ju, J. Ren and X. Qu, *Chem. Commun.*, 2013, **49**, 9791-9793.
8 Q. Peng, F. Chen, Z. Zhong and R. Zhuo, *Chem. Commun*., 2010, **46**, 5888-5890.
9 D. Pantarotto, R. Singh, D. McCarthy, M. Erhardt, J. P. Briand, M. Prato, K. Kostarelos and A. Bianco, *Angewandte Chemie*, 2004, **116**, 5354-5358.
10 A. Bianco, K. Kostarelos, C. D. Partidos and M. Prato, *Chem. Commun.*, 2005, **5**, 571-577.
11 V. Ramos-Perez, A. Cifuentes, N. Coronas, A. de Pablo and S. Borrós, *Nanomaterial Interfaces in Biology*, 2013, **1025**, 261-268.
12 B. T. Phillips, K. Gassei and K. E. Orwig, *Phil. Trans. R. Soc. B,* 2010, **365**, 1663–1678.
13 N. G. Sahoo, H. Bao, Y. Pan, M. Pal, M. Kakran, H. K. F. Cheng, L. Li and L. P. Tan, *Chem. Commun.,* 2011,**47**, 5235–5237.
14 Z. Li, Z. Wu and K. Li, *Analytical Biochemistry,* 2009, **387**, 267–270.
15 S. M. S. N. Lacerda, S. R. Batlouni, S. B. G. Silva, C. S. P. Homem and L. R. França, *Anim. Reprod.,* 2006, **3**, 146-159.
16 M. Hendricks and S. Jesuthasan, *Neural Development,* 2007, **2**, 634-642.
17 R. Badakov and A. Jaźwińska, *Cytotechnology,* 2006, **51**, 105–110.