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Combination of Polymer Technology and Carbon Nanotube Array for the Development of an Effective Drug Delivery System at Cellular Level

Cristina Riggio · Gianni Ciofani · Vittoria Raffa · Alfred Cuschieri · Silvestro Micera

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Abstract In this article, a carbon nanotube (CNT) array-based system combined with a polymer thin film is proposed as an effective drug release device directly at cellular level. The polymeric film embedded in the CNT array is described and characterized in terms of release kinetics, while in vitro assays on PC12 cell line have been performed in order to assess the efficiency and functionality of the entrapped agent (neural growth factor, NGF). PC12 cell differentiation, following incubation on the CNT array embedding the alginate delivery film, demonstrated the effectiveness of the proposed solution. The achieved results indicate that polymeric technology could be efficiently embedded in CNT array acting as drug delivery system at cellular level. The implication of this study opens several perspectives in particular in the field of neurointerfaces, combining several functions into a single platform.

Keywords Vertically aligned carbon nanotubes · Drug delivery · Alginate · NGF · PC12 cells

C. Riggio · G. Ciofani · V. Raffa · A. Cuschieri · S. Micera
Scuola Superiore Sant'Anna, Piazza Martiri della Libertà, 33, 56127 Pisa, Italy

C. Riggio (✉)
CRIM & ARTS Lab - Scuola Superiore Sant'Anna, Viale Rinaldo Piaggio, 34, 56025 Pontedera, PI, Italy
e-mail: criggio@crim.sssup.it

S. Micera
Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

Introduction

Despite advances in understanding of the mechanisms involved in the evolution of neurodegenerative disorders and neuroactive agents, drug delivery to the nervous system remains problematic, especially as accessibility to the central nervous system (CNS) is limited by the blood–brain barrier. In addition, the systemic administration of neuroactive biomolecules in order to stimulate neuronal regeneration has several limitations including toxicity and poor stability associated with many bioactive factors [1].

The purpose behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under- and overdosing. In recent years, controlled drug delivery formulations and polymers used in these systems have become much more sophisticated [2]. In addition, materials have been developed, which should lead to targeted delivery systems, in which a particular formulation can be directed to the specific cell, tissue, or site where the drug is to be delivered. Among the proposed solutions, micro- and nano-scale drug delivery systems are ideal breakthrough therapeutic approaches [3]. In this article, a carbon nanotube (CNT) array-based system, combined with a polymer thin film, is proposed as an effective drug release device directly at cellular level.

Recently, the use of carbon nanotubes [4] attracted significant attention of several groups for the development of novel neuronal interfaces [5–7]. More specifically, the electrical properties of vertically aligned carbon nanofiber (VACNFs)—a form of carbon quite similar to multi-wall CNT (MWNT)—arrays have been investigated. Two applications of this nano-device were proposed: electrical stimulation and electro-chemical sensing. In the former case, the device is configured as a forest-like VACNF array that exhibits extremely low impedance; in the latter case,

the system is designed such that the CNFs are embedded in a dielectric material (SiO_2) which should have ideal properties (low detection limits and high temporal resolution) for capturing neural signalling events.

Nguyen and collaborator also found that PC12 cells cultured on PPy-coated CNF arrays (treated with a thin layer of collagen to promote cell adhesion) can form extended neural network upon differentiation [5]. In this study, we propose a combination of drug delivery system with such CNT array, exploiting a thin film of calcium alginate as drug reservoir embedded into the platform.

Among polymers, alginate has several unique properties that have allowed it to be used as a matrix for the entrapment and/or delivery of a variety of biological agents [8]. Alginate is a co-polymer extracted from some types of brown algae and it is made up of two uronic acids: D-mannuronic acid and L-guluronic acid. Polyvalent cations are responsible for interchain and intrachain reticulations because they are tied to the polymer when two guluronic acid residuals are close [9]. The reticulation process consists of the simple substitution of sodium ions with calcium ions [10]. The relatively mild gelation process has enabled not only proteins [11], but also cells [12] and DNA [13] to be incorporated into alginate matrices with retention of full biological activity.

The polymeric film embedded in the CNT array is described and characterized in terms of release kinetics using bovine serum albumin as drug model, while in vitro assays on PC12 cell line have been performed in order to assess the efficiency and functionality of the entrapped agent (neural growth factor, NGF). PC12 cells differentiation following incubation on the CNT array embedding the alginate delivery film demonstrated the effectiveness of the proposed solution.

Materials and Methods

CNT Array: Properties, Imaging and Coating

Vertically aligned CNT arrays were provided from Nano-Lab, Inc. (Newton, MA, USA). They were grown by plasma-enhanced chemical vapour deposition (PECVD) using Ni catalyst deposited on a 200-nm thick Cr film covering a Si wafer. The average diameter of the individual CNT is 80 ± 10 nm and the height is approximately 7 μm, as specified by the supplier. The CNTs are randomly distributed in the array (1 cm × 1 cm) with a density of $8 \pm 1 \times 10^8/cm^2$. All the samples were pre-treated in 1.0 M HNO_3 for 30 min to remove the metal catalyst, and then thoroughly rinsed with deionized water. The sample was allowed to dry in air and sterilized with UV exposition before cell culture experiments.

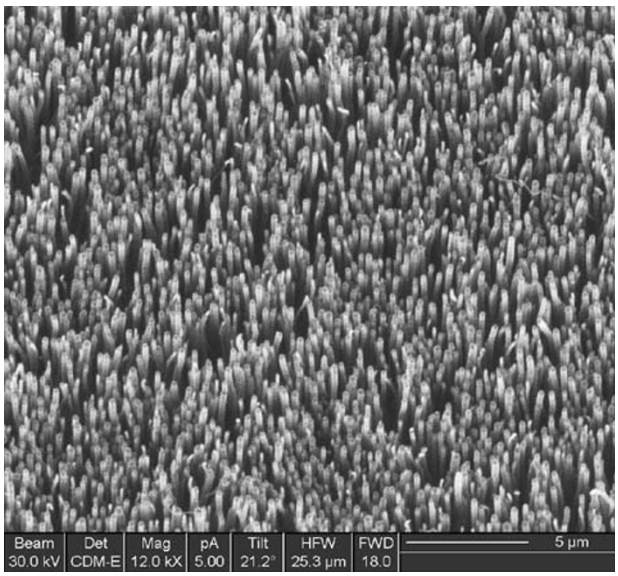

Fig. 1 FIB image of the as-grown CNT array

Figure 1 shows a focused ion beam (FIB) image of an as-grown CNT array used in this study. The FIB system used in the present study is a FEI 200 (Focused Ion Beam Localized milling and deposition) delivering a 30-keV beam of gallium ions (Ga^+).

Due to the high aspect ratio (>70:1), the as-grown CNT array is not stable when treated in liquid environments: during the drying process, CNTs irreversibly stick together to form microbundles, driven by the capillary force of water droplets. In order to prevent the CNT sticking in a liquid environment, and to improve mechanical features of CNTs, a thin layer of SiO_2 is deposited onto the array [5]. SiO_2 film was deposited via sputtering at a sputtering rate of 1 nm/min for 45 min (RF Sputtering Sistec, model DCC 150, operating at a constant pressure of 1 Pa, using 99.99% pure SiO_2 target and 99.999% pure argon as sputtering gas).

Alginate Thin Film Design, Production and Characterization

The CNT array owns a forest-like structure that could be exploited for the deposition of a polymeric thin film acting as drug delivery device.

For drug release kinetics investigation, bovine serum albumin (BSA, A3156 from Sigma, MW = 66,430 g/mol) was added to an alginate solution at a final concentration of 200 μg/mL. BSA was used as "protein model", as its molecular weight is similar to that one of NGF (N1408 from Sigma, reconstituted in a 0.1% BSA solution in PBS) and its concentration can be much more easily quantified [14]. For release kinetics investigation, the alginate solution (200 μg/mL of sodium alginate and 200 μg/mL of

BSA) was deposited onto a polystyrene clean surface at a concentration of 130 μL/cm^2 and the sample was allowed to dry under laminar flux for 12 h until the film was completely dried. Crosslinking was thus performed with a 30% $CaCl_2$ solution at a concentration of 130 μL/cm^2, gently stirred and quickly removed [15]. Three ml of distilled water was added on the polymeric film as release bulk. BSA concentration was thereafter assessed in the release bulk via spectrophotometry (with a LIBRA S12 Spectrophotometer UV/Vis/NIR, Biochrom) at 280 nm [16]. All the experiments were performed in triplicate.

Fitting of experimental data was performed with Matlab® Curve fitting toolbox, with a non-linear least square method adopting Gauss–Newton algorithm.

Cell Culture and In Vitro Testing

In vitro experiments were carried out on PC12 cells (ATCC CRL-1721), a cell line derived from a transplantable rat pheochromocytoma that responds reversibly to NGF by inducing a neuronal phenotype. In its presence, these cells undergo a dramatic change in phenotype whereby they acquire most of the characteristic properties of sympathetic neurons. Other salient responses to NGF include cessation of proliferation, generation of long neurites, acquisition of electrical excitability, hypertrophy and a number of changes in composition associated with acquisition of a neuronal phenotype [17].

PC12 cells were cultured in Dulbecco's modified Eagle's medium with 10% horse serum, 5% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Just 2% of fetal bovine serum was used for the differentiation experiments. Cells were maintained at 37 °C in a saturated humidity atmosphere of 95% air/5% CO_2.

Alginate film coated on the CNT array and entrapping NGF was tested on PC12 cells monitoring their differentiation. An alginate solution (200 μg/mL) entrapping 2 nM of NGF (N1408 from Sigma, reconstituted in a 0.1% BSA solution in PBS) was casted on the CNT array and then crosslinked with a 30% $CaCl_2$ solution as previously reported for drug release assessment. PC12 cells were seeded on an ad hoc polystyrene substrate, fabricated with high precision milling machine, at a density of 50,000/cm^2. The substrate was thereafter placed on the CNT array system and the cells were grown in differentiating medium.

Cells' images were obtained by a microscope (TE2000U, Nikon) equipped with a cooled CCD camera (DS-5MC USB2, Nikon) and with NIS Elements imaging software.

Number of cells and neurite length have been monitored with the image analysis software "ImageJ" (freely downloadable from the National Institutes of Health at http://rsb.info.nih.gov/ij/).

Results and Discussion

In Fig. 2, the scheme of the CNT array-based system for drug delivery proposed in this study is depicted. The main structure is composed by the CNT array, embedded with the thin film of alginate entrapping NGF to induce cell differentiation.

SiO_2 Coating

Figure 3a shows how as-grown CNTs stick together to form microbundles as a result of evaporation following exposition in a liquid environment. This phenomenon is completely avoided by performing an SiO_2 coating. The SiO_2 thin film, in fact, improves CNT mechanical features against the capillary force of water droplets during the drying process, thus preserving vertical alignment (Fig. 3b). High magnification (50 kX) FIB imaging reveals a non-uniform coating, having on the tips a higher thickness than at the walls (about 40 ± 2 nm at CNT tip and CNT base, 6 ± 1 nm at the wall).

Alginate Film Properties

In order to define a thickness of the film polymer comparable to the height of CNTs, different alginate solutions at several concentrations were tested producing films on Si-clean surface. Subsequently, via FIB analysis, the film thicknesses for the different conditions were measured, and finally the alginate concentration corresponding to a film thickness of approximately 5 μm was chosen.

The typical temporal trend of the protein release from the alginate thin films is reported in Fig. 4. The protein amount is given as percentage of the initial amount entrapped into the film (200 μg/cm^3 of film). The trend is well fitted ($R^2 = 97.65\%$) with a bi-exponential curve as already reported for alginate fibers [18] and microspheres [19] and described by the following expression:

$$C_2(t) = \frac{C_{10}}{1+\frac{V_2}{V_1}} \cdot \left(1 - e^{-h \cdot S \cdot \left(\frac{1}{V_1}+\frac{1}{V_2}\right) \cdot t}\right) + \frac{S \cdot C_{s0}}{V_2} \cdot \left(1 - e^{-2 \cdot k_S \cdot t}\right) \quad (1)$$

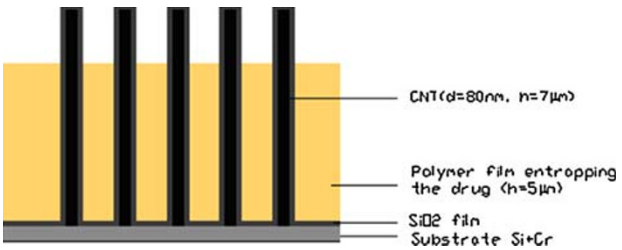

Fig. 2 Schematic illustration of the proposed CNT-based system

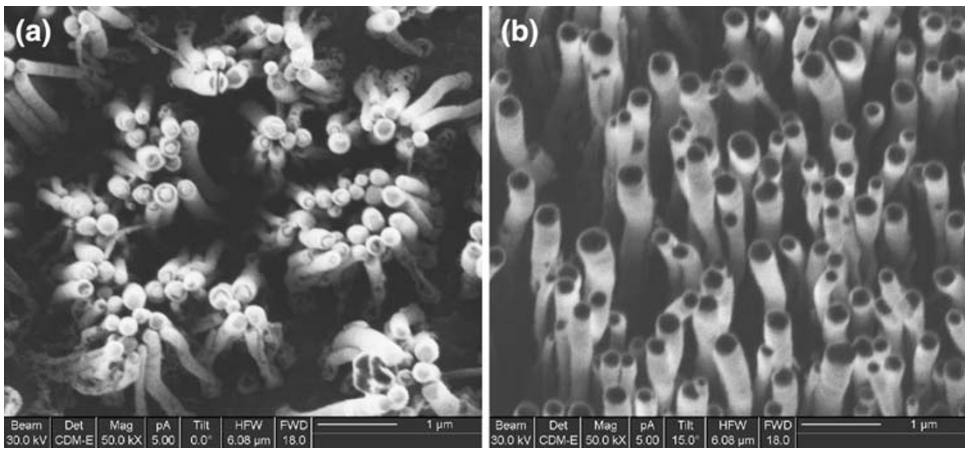

Fig. 3 Bare (**a**), and SiO_2-coated (**b**) CNT array (samples dipped in water and dried in air before the imaging)

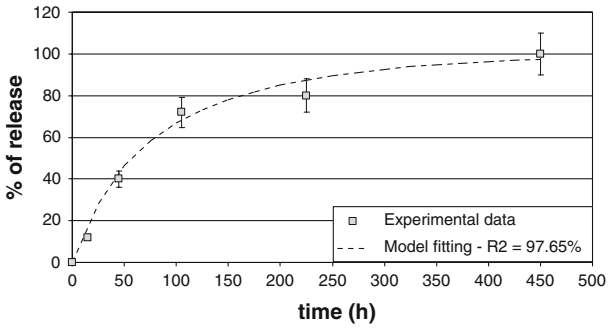

Fig. 4 Alginate release profile: experimental data and model fitting ($n = 3$)

where C_2 is the protein in the bulk, C_{10} is the concentration inside the gel, S and V_1 are, respectively, the surface and the volume of film, V_2 is the volume of the bulk, h is the massive exchange coefficient, C_{s0} is the protein concentration on the surface of the film and finally k_s is the desorption rate constant.

Substituting known values and by fitting the experimental data with the mathematical model of Eq. 1, the h value resulted 10^{-9} m/s, in agreement with data given in the literature for alginate microsphere [20].

Induction of Cell Differentiation

In vitro tests were performed in order to demonstrate that proteins entrapped in CNT array are successfully released in cell medium and fully retain their biological activity.

Figure 5 shows clearly differentiated PC12 cells after incubation on the CNT array coated with the releasing film, as described in section "Cell Culture and In Vitro Testing" The microscope analysis was carried out up to three days of incubation, and, specifically, after 8 (Fig. 5a), 24 (Fig. 5b), 48 (Fig. 5c) and finally after 72 h (Fig. 5d). Number of differentiated cells incremented during the time: at the third day of culture, the PC12 cells generate a neural network that is a demonstration that the NGF is completely released from the film and still maintains its bioactivity.

Figure 6a and b show, respectively, the percentage of differentiated cells and the neurite length at the different time points. Figure 6a shows that already after 8 h, not a negligible number of cells (about 10%) are differentiated. After 24 h, there is a spread of the number of differentiated cells, being about 85% of the total cells. In the second day, the number increased up to 90% and, in the third day, about 96% of the cells have well-developed neurites. Figure 6b reports the trend of neurite length in the time: already after 24 h, the mean length of the neurite is 33.1 ± 17.9 µm and after 72 h, the length increases up to 27.7 ± 15.9 µm.

These data do not significantly differ ($P > 0.1$, Student's t-test) from control tests performed with "free" NGF (80 ng/mL in the culture medium) where, after three days of incubation, almost 95% of the cells were differentiated with an average neurite length of about 30 µm (data not shown).

Conclusions

In this article, the authors demonstrated that a thin polymeric film-based drug delivery system can be combined to a CNT array and efficiently exploited for biomedical applications.

The system proposed in this study was developed by deposing a thin film of SiO_2 onto a CNT array in order to prevent the CNT sticking in a liquid environment. A thin film of alginate containing NGF was thereafter deposited on the CNT array. The polymer fills the array for few microns (~5 µm) allowing the CNTs to expose their tips to the microenvironment. We showed that PC12 cells—cultured on ad hoc substrate and positioned on the array—differentiated thanks to the protein released from the polymer embedded in the interface.

The results achieved indicate that polymer technology could be efficiently embedded in CNT array [21] acting as

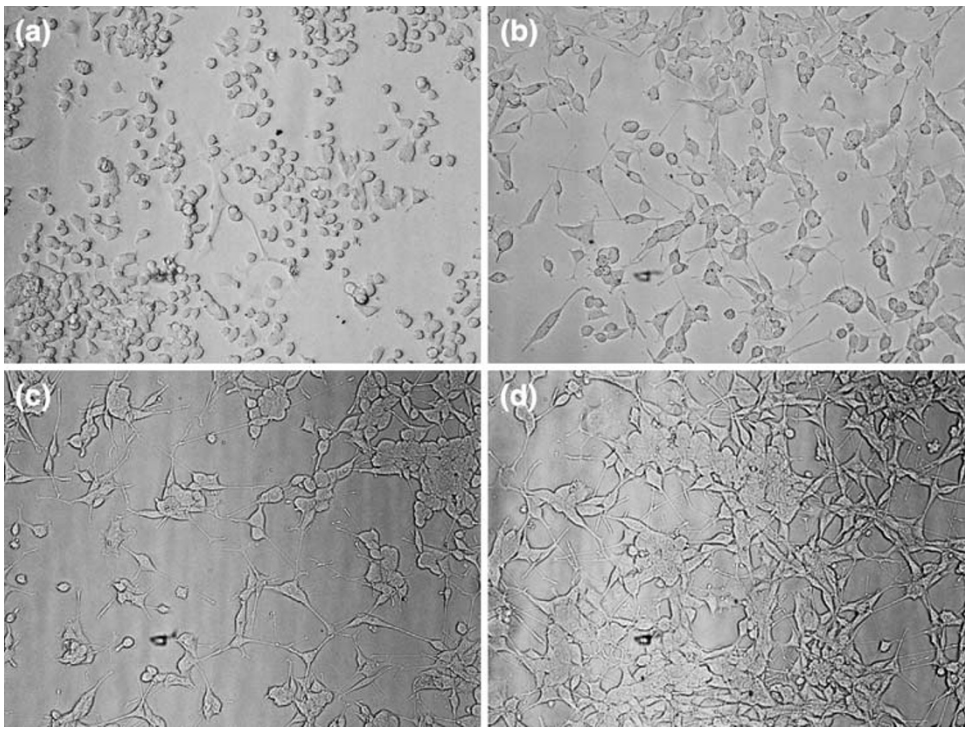

Fig. 5 Differentiated PC12 after 8 (**a**), 24 (**b**), 48 (**c**) and 72 h (**d**)

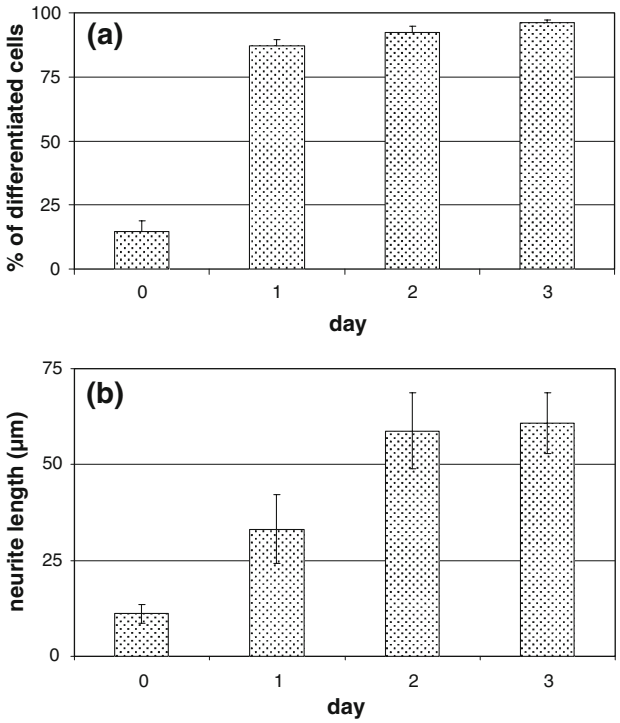

Fig. 6 Percentage of differentiated cells (**a**) and neurite length (**b**) versus time ($n = 3$)

drug delivery system at cellular level. The implication of this study opens several perspectives in particular in the field of neurointerfaces, combining several functions into a single platform [22, 23]. The nanostructured architecture of CNTs presents features that could mimic the biological complexity of the nervous system, making them suitable for clinical applications. Electrical properties could enable neural stimulation and signal recording at cellular level or offer an exciting test-bench to study the cellular behaviour at the neuronal interface. Finally, CNT-based interfaces, as demonstrated, could be used for controlled drug delivery: any bioactive factor could be released in a spatially and temporally controlled manner.

The proposed approach represents an interesting solution for building an innovative neuronal interface that could provide record of activity and/or stimulation of the nervous tissue as well as delivery of therapeutic agents at cellular level.

Although neuronal interfaces have reached clinical utility, reducing the size of the bioelectrical interface in order to minimize damage to neural tissue and maximize selectivity is still most problematic. Moreover, the efficacy of any clinical applications is ultimately determined by the quality of the neuron–electrode interface. Recently, new insights are emerging about the interactions between brain cells and carbon nanotubes, which could eventually lead to the development of nanoengineered neural devices [24]. Very interestingly, reports show that nanotubes can sustain and promote neuronal electrical activity in networks of cultured cells, by favouring electrical shortcuts between the proximal and distal compartments of the neuron [25]. The strategy of the proposed study has the possibility to couple one interface with enhanced electrical functionality with a

system for the release of neurotrophic factors. It is well proven, in fact, that biomolecular therapy is a well-established methodology for stimulation of nerve regeneration [26]. We have demonstrated the potential of polymeric, neurotrophin-eluting hydrogels to be incorporated into existing neural prosthesis designs, to improve the conditions of surrounding cells and, eventually, of the tissue-electrode interface in case of in vivo applications. In future, enabling bionanotechnology should open new perspectives in the design of the NI, allowing the integration of multi-sites for specific and simultaneous tasks with high spatial resolution [27].

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