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DESIGN OF FIBERS SPUN FROM
CARBON NANOTUBE-SPHERE BINARY COLLOIDAL SYSTEMS
AS SUBSTRATES FOR CELL BEHAVIOUR CONTROL

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THÈSE PRÉSENTÉE EN VUE DE L'OBTENTION
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Cette thèse intitulée:

DESIGN OF FIBERS SPUN FROM
CARBON NANOTUBE-SPHERE BINARY COLLOIDAL SYSTEMS
AS SUBSTRATES FOR CELL BEHAVIOUR CONTROL

présentée par: **POLIZU Stefania**

en vue de l'obtention du diplôme de : **Philosophiæ Doctor**

a été dûment acceptée par le jury d'examen constitué de:

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M. **POULIN Philippe**, Ph.D., membre et codirecteur de recherche

M. **MATEESCU Mircea**, Ph.D., membre

M. **FAVIS Basil**, Ph.D., membre

DEDICATION

This work is dedicated to my sister Alexandrina with the hope that one day the life of

people suffering from a degenerative disease will be less difficult!

Motto:

Many of (biological) cells are very tiny, but they are active; they
manufacture substances; they walk around; they wiggle; and they do
all kind of marvellous things – all on very small scale. Also they
store information. Consider the possibility that we too can make a
thing very small, which does what we want – and that we can
manufacture an object that manoeuvres at that level."

Richard P. Feynman-1959,

ACNOWLEDGEMENTS

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I would like to express my sincere gratitude to all the people who supported me and this project, family, teachers, researchers, friends and colleagues.

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To my co-supervisor, Dr. Poulin, for his warm welcome in the world of nanotubes which is enormous, marvellous, in spite of the nanoscopic dimension of these entities, my

grateful thanks! Apart from motivating scientific environment I am thankful for the french atmosphere in the lab and the outcome of our experiments.

To Dr. Rouabhia, I can't express how your interest to this project was important for me and for the accomplishments in my thesis. Thank you so much!

To Dr. Savard, I would like address my thanks for accepting to be president of the jury.

To Dr. Mateescu, thank you to be part of the jury and assume the role of the external member.

I am sincerely grateful for the participation of Dr. Favis, as member of the jury, from École Polytechnique de Montréal.

To Dr. Shirazi, thank you to participate at the jury as the Dean's representative, École Polytechnique de Montréal.

Thanks to all my friends and colleagues for their support in the easy time and difficult moments.

An important part of experimental work of this thesis was accomplished at Bordeaux, in Centre de Recherche Paul Pascal. I would have never accomplished such a work without the professional support of Maryse, Alain and the other members of the Dr. Poulin's research group. Thank you all!

The time I lived at Bordeaux would have been much more difficult in the absence of all facilities offered by the laboratory. Thank you!

Sincere thanks to Laetitia, Anouk and Alix for their friendly welcome every time, to help me enjoy the weekends, when I was far from my familiy. I will always remember the good moments.

Sinziana, Cristiana and Viorel how to say how much I love you and how your love strengthened me in the difficult moments during the past years. You, my Friends and my family, were would I have been without you! Thank you for every thing!

RÉSUMÉ

Le travail de cette thèse est consacré à la conception de nouvelles fibres contenant des nanotubes de carbone (NTCs) et à l'évaluation de ces fibres nano-structurées et proposées comme substrat pour la croissance des cellules neuronales. Des travaux de recherche précédents ont confirmé la possibilité de filler des fibres à partir de suspensions aqueuses de nanotubes de carbone mono-parois (SWNTs) ou de nanotubes multi-parois (MWNTs). La méthode de filage proposée, la coagulation des particules colloïdales, (PCS) permet la fabrication des fibres à partir de particules colloïdales : des nanotubes dispersés dans l'eau, stabilisés par des agents tensio-actifs sont alors injectés dans un écoulement d'une solution externe composée d'un polymère de coagulation. Cette technique s'avère très avantageuse pour la fabrication d'un biomatériau à base de nanotubes de carbone. Cependant, jusqu'à présent, aucune nouvelle alternative à base de nanotube de carbone n'a été proposée pour la fabrication des biomateriaux comme support pour la croissance de cellules neuronale.

L'objectif principal de ce travail de recherche est de concevoir des biomatériaux neuronaux à base de nanotubes de carbone organisés sous forme de fibres macroscopiques. En utilisant le principe du filage humide, nous proposons une approche hybride permettant l'intégration des nanotubes de carbone (NTCs) en fibres macroscopiques biocompatibles et biodégradables. Par cette nouvelle méthode de fabrication basée sur le processus de filage humide, nous évitons l'utilisation de la

modification chimique covalente des NTCs, ce qui permet de préserver les caractéristiques intrinsèques des nanotubes. Notre concept est basé sur le développement des Systèmes Colloïdaux Binaires de Nanotube-Sphère (NSBCS), filables dans un processus de filage humide. Ils contiennent des nanotubes de carbone dispersés avec du dodécyl sulfate de sodium (SDS) et une suspension aqueuse de nanoparticules de copolymère d'acides polylactique-co-glycoliques, (PLGA) qui sont mélangés dans des proportions différentes. L'efficacité de cette méthode réside dans l'effet synergique des nanoparticules sphériques et des particules bâtonnets assemblées dans un système colloïdal binaire, qui joue un rôle primordial dans le processus de filage.

Les fibres macroscopiques que nous produisons sont une combinaison harmonieuse de nanotubes de carbone dispersés, de copolymère PLGA et d'alcool polyvinylique (PVA). Par leur incorporation, les nanoparticules de PLGA soutiennent la structure des fibres, permettant ainsi l'arrangement des nanotubes dans un réseau de nanoparticules. Une contribution supplémentaire attribuée à l'action du PLGA en complémentarité avec le PVA améliore considérablement le processus de fabrication. L'introduction de la suspension de PLGA est critique pour ce travail car par leur l'incorporation, les nanoparticules sphériques limitent l'apparition de la phase de séparation.

La configuration des dispersions mélangées au niveau sous-microscopique et microscopique est liée à l'emprisonnement spatial engendré par les espaces créés par l'organisation des particules sphériques. Elles assurent l'insertion des nanotubes dans le

treillis de PLGA et contribuent à la formation de la fibre. Cette restriction spatiale induite par l'insertion des nanotubes parmi les nanoparticules est augmentée d'avantage par le passage du mélange de l'aiguille cylindrique de la seringue à la buse conique, durant l'injection du mélange dans le bain de coagulation. À cette étape du processus, la combinaison de l'effet d'emprisonnement avec l'action d'écoulement est avantageuse.

La méthode que nous proposons favorise le filage des fibres macroscopiques à partir de ce système binaire, consistant en un mélange colloïdal de nanotubes et de nanoparticules de PLGA en proportions variables. En proposant le filage à partir d'un système colloïdal binaire, nous produisons de nouvelles conditions pour le mécanisme de coagulation. Le mélange colloïdal, à son entrée dans le bain de coagulation, est engagé dans le processus de coagulation qui se développe en deux étapes: i) la première est le contact avec la solution contrôlé par l'adsorption de PVA sur PLGA dû à leur affinité; ii) le pontage entre nanotubes, les nanosphères et les chaînes de PVA suit et le phénomène de coagulation se développe. Comme résultat, un mélange tertiaire est formé et il est organisé dans un mono-filament avec une texture variable et une structure intérieure très particulière.

Ces fibres varient dans leur composition et possèdent une morphologie particulière qui augmente leur capacité de stimuler et soutenir la prolifération de cellules, en particulier les cellules neurales, tout en offrant un support efficace pour la différentiation de ces cellules. Ces structures sont idéalement adaptées pour diriger la croissance cellulaire. En

proposant l'introduction de la suspension des nanoparticules de PLGA, nous travaillons sur les caractéristiques des nouvelles fibres, en particulier leur biodégradabilité et leur biocompatibilité. Grâce au PLGA, les fibres deviennent partiellement biodégradables, ce qui est un accomplissement très important, considérant que la biodégradabilité en conditions physiologiques est une limitation pour les NTCs et le PVA. Le processus de dégradation provoque une structure fibrillaire dans laquelle le NTC forme un réseau qui réduit les effets de libération des nanotubes, un point critique pour leur biocompatibilité à long terme. Ainsi, la permanence des nanotubes dans un réseau structuré augmente le contact avec les cellules et maintient leur biofunctionalité pendant et après la dégradation des fibres. Cette approche hybride augmente considérablement l'efficacité des NTCs, de ce fait augmentant la biocompatibilité du matériel et sa capacité d'agir. L'adhérence de cellules est fortement liée à la présence des nanotubes sur la surface et on peut distinguer un lien étroit entre l'organisation des nanotubes et la morphogénèse des cellules. Dans une telle armature, les nanotubes, possédant une surface spécifique élevée, deviennent très sensibles à la croissance de cellules et incitent leur différentiation. Cette dernière est exprimée par la formation de neurites et leur prolongation, même en l'absence des facteurs de croissance (NGFs), un stimulateur pour la différentiation. La surface est texturisée avec un ordre périodique et augmente le contact avec les cellules. Chaque fibre possède des caractéristiques spécifiques issues de la formulation. L'organisation structurale de la fibre sous une forme allongée, au niveau nano, micro- et macromoléculaire, est semblable à la structure fibrillaire de la matrice

extracellulaire et imite le réseau de neurones, de ce fait favorisant l'interaction avec les cellules.

Les caractéristiques mécaniques des nouvelles fibres à base de nanotubes monoparois (SWNTs) sont un accomplissement majeur dans la conception des fibres par l'approche hybride et démontrent une bonne corrélation entre les propriétés mécaniques et les caractéristiques structurales. Leur élasticité et flexibilité ont des conséquences positives sur les interactions avec les cellules nerveuses, sachant que les substrats flexibles établissent un contact approprié avec les cellules nerveuses. Leur effet synergique commande la réponse cellulaire par l'interface entre cellule et substrat. L'analyse chimique de la surface par TOF-SIMS complétée par la composition de phase, visualisée par AFM, indique une distribution homogène des différents composants sur la surface des fibres. Une telle composition homogène est en accord avec d'autres caractéristiques de la surface.

Nous proposons une approche hybride pour produire des fibres à base de NTCs en tant que biomatériau pour des applications neuronales. Les caractéristiques de ces fibres, tel que déterminées par cette étude, démontrent la validité de la nouvelle méthode pour la conception de nouveaux substrats fibrillaires pour soutenir la croissance des cellules. Par ailleurs, l'utilisation d'une solution de coagulation aqueuse confère la propreté du matériel et permet l'introduction de nombreux agents actifs ou de molécules biologiques

sans leur dénaturation. C'est une opportunité pour l'application de pré- ou post-traitements afin de fabriquer des biomatériaux hybrides complexes contenant des NTCs.

Le futur de ces fibres est prometteur et nous envisageons leur utilisation pour des dispositifs conçus pour la régénération de la moelle épinière. Ce sont les premières fibres à base de nanotubes de carbone produites par une approche hybride utilisant le processus de filage humide dans une solution aqueuse. Elles sont biocompatibles et biodégradables.

ABSTRACT

The research presented in this thesis focuses on the design of new fibers containing carbon nanotubes and the evaluation of these nanostructured fibers proposed as substrate for the control of cell' behaviour. Previous researches have confirmed the possibility to spin fibers from aqueous suspensions of single wall nanotubes (SWNTs) or multiwall nanotubes (MWNTs). The proposed Particle Coagulation Spinning (PCS) method allows the fabrication fibers using nanotubes dispersed in water, stabilised by surfactants and then injected into a coflowing stream of a coagulating polymer solution. Hence it results that, shaping CNT-biomaterials through a wet spinning process in aqueous media is very advantageous for the engineering of biomaterials. However, up till now, no new alternatives have been proposed for the design of fiber neural biomaterial as substrates for nerve growth.

The aim of this work is to design new carbon nanotube neural biomaterials shaped as fibers, where the biodegradability and biocompatibility are achieved. Capitalizing on wet spinning process, we propose a hybrid approach allowing the integration of carbon nanotubes (CNTs) in macroscopic fibers with biodegradable and biocompatible responses. This new fabrication method use the wet spinning process which eludes the CNT's covalent chemistry, thus preserving the intrinsic characteristics of nanotubes. Our

concept is based on the development of a spinnable Nanotube-Sphere Binary Colloidal System (NSBCS) for a wet spinning process. It contains CNTs dispersed with sodium dodecyl sulphate (SDS) and an aqueous suspension of polylactic-co-glycolic acid (PLGA) nanoparticles combined in a variety of ratios. The efficiency of this method resides in the synergistic effect of spherical nanoparticles and rod-like particles assembled in a binary colloid system which plays a main role in the spinning process. The macroscopic fibers we produce are a harmonized combination of dispersed CNTs, PLGA copolymer and polyvinyl alcohol (PVA). The incorporation of PLGA nanoparticles underpins the fiber structure, allowing the arrangement of nanotubes in the nanoparticles' lattice. A complementary contribution of PLGA and PVA greatly improves the fabrication process and complement the functionality of CNTs integrated in this macroscopic form.

The introduction of PLGA suspension is critical for this work. The incorporation of spherical nanoparticles avoids the phase separation behaviour and contributes to the formation of very homogenous and stable colloidal mixtures.

The configuration of mixture dispersions at sub-microscopic and microscopic level is related to the spatial confinement created by the gaps between spherical particles. They ensure the insertion of nanotubes in the PLGA lattice and assist the formation of the fiber during the spinning process. A spatial confinement is induced through the insertion of nanotubes between the nanoparticles. It is further increased by the passage of the mixture from cylindrical syringe's needle to conical nozzle, during the

injection of the mixture into the coagulation bath. At this stage of the process, the combination of the confinement effect with the shear flow action is advantageous.

The method we propose promotes the spinning of CNT macroscopic fibers from a binary colloidal mixture containing CNTs combined with PLGA nanoparticles in a variety of ratios, thus resulting in fibers with various CNT content. PLGA spherical nanoparticles root the structuring of fibers, thus improving the spinnability of the mixture for the fabrication of macroscopic threads. Moreover, by spinning from a binary colloidal system, we generate new conditions for the coagulation mechanism. The colloidal mixture prolongs its performance as a many-particle system at its entry in the coagulation bath were the coagulation emerges in two steps: i) the first contact is controlled by the adsorption of PVA on PLGA due to their affinity ii) the bridging between CNTs, nanospheres and PVA chains pursues and the real coagulation phenomena takes place. As the result, they form a tertiary blend organized in a fibrous monofilament thread with variable texture and a very particular inner structure. The new fibers do not show a skin-core structure. These fibers with variable compositions show a unique morphology that provides the capacity to stimulate and sustain cell proliferation, particularly for neural cells, while offering an efficient substrate for cell differentiation. These structures are ideally suited to direct cellular growth and for future tissue engineering applications under the form of fibers and weaved or non-woven textiles.

By initiating the suspension of PLGA nanoparticles, we tailor the characteristics of new fibers, particularly their biodegradability and their biocompatibility. Thanks to PLGA, the fibers become partially biodegradable, which is an important achievement,

considering that biodegradabilty in physiological conditions is a limitation for CNTs and PVA. The degradation process gives rise to a fibrillar structure in which CNTs form a framework-like arrangement that overwhelms the releasing effects of nanotubes, a critical point for long term biocompatibility. The permanence of nanotubes in a structured network increases the contact with cells and maintains their biofunctionality during and after the biodegradation of macroscopic fiber.

The hybrid approach greatly increases the CNTs efficiency, thus enhancing the biocompatibility of materials and their capacity to interact with cells. Cell adhesion is strongly related to the presence of nanotubes onto the surface and a strong relationship exists between the nanotubes' organisation and the cells' morphogenesis. In such a frame, the nanotubes, possessing a large specific area, become a sensitive means of stimulating cell growth and incite the differentiation of cells, expressed by the formation of neurites and their extension. The fibrous substrate promotes and sustains the formation of neurites even in the absence of Nerve Growth Factors (NGFs), a stimulator for the differentiation. Endowed with immanent characteristics, these new fibers are designed as a new substrate for neural cells, capable not only of sustaining their proliferation but also of guiding nerve growth in a preferentially controlled way.The textured surface with a periodic columnar order and particular topological features enhances the fiber contact with cells.

Each fiber possesses specific characteristics, tailored by the formulation. The fiber's structural organization in branched-lengthened form at nano-, micro- and macro-

scale is similar to the fibrillar structure of the extracellular matrix and imitates the network of neurons' organization, thus favouring the interaction with the cells.

The mechanical characteristics of SWNT-fibers complete the performance of the hybrid fibers and demonstrate the correlation between the structural characteristics and mechanical properties. The elasticity and flexibility of these fibers have positive impact on the interactions with nerve cells because the flexible fibers establish a proper contact with neural cells. Their synergistic action controls the cellular response through the cell-substrate interface.

The surface chemistry analysis by TOF-SIMS completed by the imaging with AFM in phase mode reveals a homogenous distribution of different component at the surface of fibers. Such a homogeneous composition is in accord with other surface characteristics.

We propose a hybrid approach to produce CNT-fibers as neural biomaterial. The characteristics of these fibers, as determined through this work, demonstrate the validity of this method for the design of new fibrillar substrates for cell sustaining the growth of cells. Moreover, the presence of an aqueous coagulant medium results in the material's cleanness and allows the introduction of numerous active agents or biological molecules without their denaturation. This is an opportunity for the application of pre- or post-treatments in order to manufacture complex hybrid biomaterials containing CNTs.

The future of these fibers looks promising. They are the first fibers produced by a hybrid approach using the wet spinning process. They are *in vitro* biocompatible and biodegradable.

CONDENSÉ EN FRANÇAIS

L'ouvrage de cette thèse porte sur la conception de nouvelles fibres à base de nanotubes de carbone (NTCs) ainsi que sur leur évaluation en tant que biomatériau biocompatible et biodégradable. L'objectif principal de ce travail de recherche est de concevoir des biomatériaux neuronaux à base de nanotubes de carbone organisés sous forme de fibres macroscopiques. Ces fibres nano-structurées sont proposées comme substrat pour la croissance des cellules neuronales.

Dans cette perspective, nous avons identifié le processus de filage comme étant idéal pour l'incorporation des structures de nanotube de carbone dans une forme macroscopique. En utilisant le principe de filage humide, nous proposons une approche hybride permettant l'intégration des nanotubes de carbone (NTCs) en fibres macroscopiques capables de guider préférentiellement la croissance des cellules neuronales et stimuler leurs fonctions. L'approche hybride consiste en le mélange des NTCs avec une composante organique tel que le copolymère. Dans la même lumière, la géométrie de bâtonnets des nanotubes de carbone est combinée avec la forme sphérique des nanoparticules de polymère afin de créer de nouvelles structures spatiales intégrant les nanotubes et d'induire la biodégradabilité.

La nouvelle méthode de fabrication que nous proposons est basée sur le processus de filage humide permettant l'intégration de nanotubes de carbone dans une forme

macroscopique, sans faisant appel à leur modification chimique covalente. Ceci permet de préserver les caractéristiques intrinsèques des nanotubes de carbone. Notre concept est basé sur le développement des Systèmes Colloïdaux Binaires de Nanotube-Sphère (NSBCS), facilement filables dans un processus de filage humide. Ces systèmes sont préparés comme des mélanges de dispersions contenant des nanotubes de carbone dispersés avec du dodécyl sulfate de sodium (SDS) et des suspensions aqueuses de nanoparticules de copolymère d'acide polylactique-co-glycoliques, (PLGA). L'efficacité de cette méthode réside dans l'effet synergique des nanoparticules sphériques et des particules bâtonnets assemblées dans un système colloïdal binaire, qui joue un rôle primordial dans le processus de filage. Les fibres macroscopiques que nous produisons sont une combinaison harmonieuse de nanotubes de carbone dispersés, de copolymère PLGA et d'alcool polyvinylique (PVA). Pour se faire, quatre objectifs spécifiques ont été formulés :

i) Concevoir un modèle expérimental basé sur les Systèmes Colloïdaux Binaires de Nanotube-Sphère (NSBCS) utilisés en tant que mélanges pour le filage humide ;

ii) Formuler et fabriquer les fibres macroscopiques de nanotubes de carbone par le processus de filage humide;

iii) Identifier et évaluer les caractéristiques spécifiques de nouvelles fibres à base de nanotubes de carbone;

iv) Étudier le potentiel de ces fibres en tant que biomatériaux et prouver leur biocompatibilité et leur biodégradabilité en relation avec les cellules neuronales et les fibroblastes.

Pour réaliser ces objectifs, nous avons adopté une stratégie en cinq étapes permettant de valoriser les caractéristiques du système binaire colloïdale et d'exploiter au maximum le potentiel de ce processus de filage humide.

Dans la première étape, une nouvelle dispersion est conçue pour le filage qui est basée sur un mélange colloïdal contenant les nanotubes de carbone et une nouvelle composante. Nous avons identifié le copolymère de l'acide polylactique-co-glycolique (PLGA) qui est soluble en plusieurs solvant organiques et qui précipite en tant que nanoparticules sphériques suspendues dans l'eau. En faisant ce choix, nous avons considéré la biocompatibilité et la biodégradabilité comme deux caractéristiques essentielles pour atteindre nos objectifs. Le copolymère de PLGA est facilement dissous dans l'acétone, partiellement miscibles avec l'eau, de ce fait évitant le comportement de séparation de phase. La formation des nanoparticules de copolymère par nanoprécipitation est une technique bien connue dans le processus pharmaceutique et permet la précipitation du copolymère de la solution organique dans l'eau, de ce fait formant une suspension aqueuse.

La deuxième étape consiste en la préparation du mélange colloïdal binaire en mélangeant une dispersion aqueuse de NTCs avec une suspension aqueuse de nanoparticules de PLGA. Cette étape est décisive pour la conception de la structure des fibres, car les caractéristiques du copolymère sont déterminantes pour le processus de mélange. Un modèle expérimental a été établi pour les nanotubes monoparois (SWNTs) et pour les nanotubes multiparois (MWNTs), en prenant en compte le rapport de mélange entre les composantes afin d'obtenir une dispersion colloïdale stable avec une bonne capacité de filage.

La troisième étape est le développement du processus de filage qui inclut l'injection du mélange colloïdal dans le bain de coagulation suivi de la coagulation du mélange. Les caractéristiques de ce dernier influencent fortement la formation des fibres continues dans le bain de coagulation. Par cette étape, nous atteignons le deuxième objectif spécifique. L'opération de lavage des fibres en milieu aqueux est essentielle pour enlever le PVA résidu et le degré de nettoyage influence les propriétés de surface des fibres. En fait, l'insolubilité du PLGA dans l'eau est une caractéristique avantageuse qui enrichi davantage la surface de la fibre avec une composante biocompatible.
Le séchage des fibres dans des conditions ambiantes est conduit pour éliminer l'eau par une lente évaporation. L'incompatibilité du PLGA avec l'eau est aussi avantageuse dans ces conditions et contribue à la formation des fibres avec une forme circulaire.

L'accomplissement du troisième objectif spécifique réclame la caractérisation générale des fibres et les tests de biodégradabilité. Dans cette quatrième étape, nous avons identifié six caractéristiques spécifiques des fibres: la structure interne, les caractéristiques de surface, la composition, les propriétés mécaniques et le comportement visco-élastique. La biodégradabilité des fibres, qui est principalement influencée par la quantité de copolymère utilisée et par ses caractéristiques, a été prouvée.

Le travail de la cinquième étape a été dédié à l'accomplissement du quatrième objectif spécifique en faisant la preuve de la biocompatibilité des fibres. Cette étape comprend des tests in vitro, pour la réponse de fibres d'essai en contact avec les cellules neuronale, PC12, et les fibroblastes. L'utilisation des cellules PC12 est directement liée à la biofonctionalité de ces fibres tandis que le deuxième type est lié au contact de ces fibres avec la matrice cellulaire supplémentaire.

Des travaux de recherche précédents ont confirmé la possibilité de filler des fibres à partir de suspensions aqueuses de nanotubes de carbone mono-parois (SWNTs) ou de nanotubes multi-parois (MWNTs). La méthode de fabrication est basée sur la coagulation des particules colloïdales et la préparation de fibres à partir de celles-ci: des nanotubes dispersés dans l'eau, stabilisés par des agents tensio-actifs sont alors injectés dans un écoulement d'une solution externe composée d'un polymère de coagulation. Cette technique s'avère très avantageuse pour la fabrication d'un biomatériau à base de nanotubes de carbone. Par leur incorporation, les nanoparticules de PLGA soutiennent la

structure des fibres, permettant ainsi l'arrangement des nanotubes dans le réseau de nanoparticules. Une contribution supplémentaire est attribuée à l'action du PLGA en complémentarité avec le PVA, ce qui améliore considérablement le processus de fabrication. L'introduction de la suspension de PLGA est critique pour ce travail car par leur l'incorporation, les nanoparticules sphériques limitent l'apparition de la phase de séparation.

La configuration des mixtures colloïdales au niveau sous-microscopique et microscopique est liée à l'emprisonnement spatial des nanotubes engendré par les espaces créés par l'organisation des particules sphériques. Elles assurent l'insertion des NTCs dans le treillis de PLGA et contribuent à la formation de la fibre. Cette restriction spatiale induite par l'insertion des nanotubes parmi les nanoparticules est augmentée davantage par le passage de la mixture dans l'aiguille cylindrique de la seringue à la buse conique, durant l'injection du mélange dans le bain de coagulation. À cette étape du processus, la combinaison de l'effet d'emprisonnement avec l'action d'écoulement est avantageuse.

En proposant le filage à partir d'un système colloïdal binaire, nous engendrons de nouvelles conditions pour le mécanisme de coagulation. Le mélange colloïdal, à son entrée dans le bain de coagulation, est engagé dans le processus de coagulation qui se développe en deux étapes: i) la première est le contact avec la solution contrôlé par l'adsorption de PVA sur PLGA dû à leur affinité; ii) le pontage entre nanotubes, les

nanosphères et les chaînes de PVA suit et le phénomène de coagulation se développe. Comme résultat, un mélange tertiaire est formé et il est organisé dans un mono-filament avec une texture variable et une structure intérieure très particulières.

Ces fibres varient dans leur composition et possèdent une morphologie originale qui augmente leur capacité de stimuler et soutenir la prolifération de cellules, en particulier les cellules neuronales, tout en offrant un support efficace pour la différentiation de ces cellules. Ces structures sont idéalement adaptées pour diriger la croissance cellulaire. En proposant l'introduction de la suspension des nanoparticules de PLGA, nous travaillons sur les caractéristiques des nouvelles fibres, en particulier leur biodégradabilité et leur biocompatibilité. Grâce au PLGA, les fibres deviennent partiellement biodégradables, ce qui est un accomplissement très important, considérant que la biodégradabilité en conditions physiologiques est une limitation pour les CNTs et le PVA. Le processus de dégradation provoque une structure fibrillaire dans laquelle les NTCs forment un réseau qui réduit les effets de libération des nanotubes, un point critique pour leur biocompatibilité à long terme. Ainsi, la permanence des nanotubes dans un réseau structuré augmente le contact avec les cellules et maintient leur biofonctionalité pendant et après la dégradation des fibres. Cette approche hybride augmente considérablement l'efficacité des NTCs, de ce fait augmentant la biocompatibilité du matériel et sa capacité d'agir. L'adhérence de cellules est fortement liée à la présence des nanotubes sur la surface et on peut distinguer un lien étroit entre l'organisation des nanotubes et la morphogénèse des cellules. Dans une telle armature, les nanotubes, possédant une

surface spécifique élevée, deviennent très sensibles à la croissance de cellules et incitent leur différentiation. Cette dernière est exprimée par la formation de neurites et par leur prolongation, même en l'absence des facteurs de croissance (NGFs), un stimulateur pour la différentiation. La surface est texturisée avec un ordre périodique, ce qui augmente le contact avec les cellules. Chaque fibre possède des caractéristiques spécifiques issues de la formulation. L'organisation structurale de la fibre sous une forme allongée, à des niveaux nano, micro- et macromoléculaires, est semblable à la structure fibrillaire de la matrice extracellulaire et imite le réseau de neurones, de ce fait favorisant l'interaction avec les cellules.

Les caractéristiques mécaniques des nouvelles fibres à base de nanotubes monoparois (SWNTs) sont un accomplissement majeur dans la conception des fibres par l'approche hybride et démontrent une corrélation entre les propriétés mécaniques et les caractéristiques structurales. La résistance à la rupture, le module d'élasticité et la déformation sont les trois caractéristiques mesurées. Leurs valeurs prouvent la continuité des fibres et démontre une intégration efficace des nanotubes à l'intérieur de la fibre. La flexibilité des fibres est une autre caractéristique avec conséquences positives sur les interactions avec les cellules nerveuses, sachant que les substrats flexibles établissent un contact approprié avec les cellules nerveuses. Leur effet synergique commande la réponse cellulaire par l'interface entre cellule et substrat.

Le comportement viscoélastique des fibres a été testé en fonction de la température et les résultats obtenus sont en accord avec les caractéristiques mécaniques. Les valeurs du

module élastique, du module de perte et du facteur de perte mettent en évidence la contribution du copolymère à la formation de nouvelles fibres. L'apparition de nouvelles transitions de phase démontre l'existence des interactions entre les composantes des fibres.

L'analyse chimique de la surface effectuée par la technique TOF-SIMS complétée par la distribution de phase, visualisée par AFM, indique une distribution homogène des différents composants sur la surface des fibres. Une telle composition homogène est en accord avec d'autres caractéristiques de la surface, tel que la morphologie et la topographie.

Les caractéristiques de ces fibres, telles que déterminées par cette étude, démontrent la validité de la nouvelle méthode pour la conception de nouveaux substrats fibrillaires pour soutenir la croissance de cellules neuronales. Par ailleurs, l'utilisation d'une solution de coagulation aqueuse confère la propreté du matériel et permet l'introduction de nombreux agents actifs ou de molécules biologiques sans leur dénaturation. Ce fait est une opportunité pour l'application de pré- ou post-traitements afin de fabriquer des biomatériaux hybrides complexes contenant des NTCs.

Le futur de ces fibres est prometteur et nous envisageons leur utilisation pour des dispositifs conçus pour la régénération de la moelle épinière. Ce sont les premières fibres à base de nanotubes de carbone produites par une approche hybride utilisant le processus de filage humide dans une solution aqueuse. Elles sont biocompatibles et biodégradables.

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LIST OF ABREVIATIONS

Ch - Chiral vector

θ - Chiral angle

CMC - critical micelle concentration

CNTs-carbon nanotubes

CS-Cross section

CNS-Central Nervous System

DLS – Dynamic Light Scattering

DMA - Dynamic Mechanical Analysis

E - Elastic Modulus

FDA- Food and Drug Administration

FM - Fluorescence Microscopy

G'- Storage Modulus

G''- Loss Modulus

GPa – giga Pascal

MEMS – Micro Electro Mechanical Systems

MPa – mega Pascal

MTT- Test for *in vitro* Viability of Cells

MWNT – multi wall carbon nanotubes

NGFs - neural growth factors

NSBCS -Nanotube-Sphere Binary Colloidal System

OD-Optical Density

OM-Optical Microscope

Pa – pascal

PBS - phosphor buffer saline

PCS – Particle Coagulation Spinning

PC12 - rat pheochromocytoma cells

PNS-Periferic nervous system

PVA – Poly vinyl alcohol

PLGA-Poly-lactic-co-glycolic acid

RG503H-end group uncapped Resomer Copolymer

RG502- end group capped Resomer Copolymer

RMS - root mean squared

SDS - Sodium Dodecyl Sulphate

SEM – Scanning Electron Microscopy

SWNT- single wall carbon nanotubes

Tan δ- Loss factor

TOF-SIMS – Time of Flight Secondary Ion Mass Spectroscopy

TGA-Termogravimetric Analysis

T_g - Glass Transition Temperature (^{o}C)

T - Temperature (^{o}C)

GENERAL INTRODUCTION

In the world of nanotechnology, the ability to build various new systems based on atomic and molecular units provides the field of material engineering with distinct advantages over its conventional potential. When these resources are oriented towards biomedical and biotechnological applications, it becomes possible to construct innovative supramolecular architectures with greater flexibility and precise response and to engineer biomaterials with properties similar to those of cellular biologic microenvironment.

Biomaterials are an important domain of application for nanotechnology. Continuous progress in nanomaterials and nanostructures conjugated with the development of nanoscience and nanotechnology becomes visible in the new achievements in this field. As new structures, carbon nanotubes (CNTs) motivate the creation of new biomaterials and components for miniaturized medical devices. Due to their small size, large surface area and high aspect ratio, these structures demonstrate outstanding mechanical characteristics and a unique electrical conductivity. In contact with a biological environment, they offer a nanoscale response with an enormous potential for interactions with cells at a molecular level.

The emerging technology of CNTs and their suitability for biological and medical applications have marked a new paradigm. We are now faced with new

biocompatibility perspectives while a great challenge is associated with the integration of carbon of nanotubes (CNTs) in micrometer and macroscopic structures, without loosing the outstanding properties conferred by their nanoscopic dimension. In 2004, two studies have shown that the handling of the CNT's pristine is associated with potential toxicological effects. Since, the issue of size and shape has seeded a big controversy regarding the quest of CNTs and their process. Facing this dilemma, the incorporation of CNT structures into macroscopic materials through a hybrid approach is proposed as a possible response to this double faced problem. This research is important in order to provide safe CNT-based biomaterials. The fabrication of new macroscopic CNT-fibers able to preserve the intrinsic characteristics of nanotubes is a great achievement. An optimal transfer of these characteristics from a nanometric level to a macroscpic form is revealed by the performances of fibers.

A new concept based on the development of a spinnable Nanotube-Sphere Binary Colloidal System (NSBCS) is proposed and guarantees the successful fabrication of new CNT-fibers by a wet spinning process. This colloidal mixture contains CNTs dispersed with sodium dodecyl sulphate (SDS) and an aqueous suspension of polylactic-co-glycolic acid (PLGA) nanoparticles combined in a variety of ratios. After the injection of this mixture in a coagulating solution of polyvinyl alcohol (PVA), the coagulation process takes place and a monofilament thread is constructed. The efficiency of this method resides in the synergistic effect of spherical nanoparticles and nanotubes assembled in a binary colloid system. The resulting macroscopic fibers have

various compositions consisting of CNTs, PLGA and PVA. The introduction of PLGA nanoparticles, as a component of the colloidal mixture, is the key stone of this work. They endow the fibres with a biodegradable and biocompatible behaviour, as main requirements for a successful biomaterial. Designed as neural biomaterials, these fibers have the capacity to support the growth of neuronal cells, to stimulate their proliferation and differentiation and to incite their migration. The content of this thesis emphasizes the formulation of new CNTs-based fibers with biocompatible and biodegradable behaviour, as well as their fabrication and evaluation of their specific characteristics. The outline of the content is as follows:

Chapter one includes a literature review of the state of the art of carbon nanotubes as biomaterials with reference to their structure, properties and synthesis. The potential of carbon nanotubes for biomedical applications is largely explored. This extensive review is completed by a brief presentation of the polylactic-co-glycolic acid copolymers and polyvinyl alcohol, as biomaterials and as components for the fiber's formulation. A brief description of nerve cells along with the issue of nerve regeneration closes the first chapter.

Chapter two presents the general goal of this study and the specific objectives of this research work. A description of the adopted strategy for the accomplishment of these objectives is included. The content of this thesis includes four papers, each of which has a different topic. The correlation between these papers aims to integrate each part of the work to the general goal of this thesis.

Chapter three focuses the experimental aspects of this study. The presentation of the experimental techniques employed to accomplish the experimental work along with the experimental protocols are included.

Chapter four emphasizes the innovative aspects of this thesis and presents the hybrid approach for the formulation of biomaterial and its fabrication as macroscopic CNT-fibers.

Chapter five investigates the specific characteristics of new SWNT-fibers. A strong relationship between the processes, the structure and its properties is schown through this investigation.

Chapter six focuses on the nanoscale surface characteristics of CNT-fibers in the perspective of their role played in the interaction with living cells.

The general discussion presented in Chapter seven focuses on the novelty of this project and the multiple aspects related to the fiber's design and fabrication. The main characteristics of fibers are shown as a result of the new formulation. The fibers performances are evaluated from a biomaterial perspective.

A CONCLUSION ON THIS RESEARCH EVIDENCES THE MAIN CONTRIBUTIONS IN RELATION TO THE CHALLENGES IN THIS FIELD. A PERSPECTIVE OF NEW RESEARCH PROJECTS IS PROPOSED.

CHAPTER 1

LITERATURE REVIEW

1.1. CNT FOR BIOMATERIALS: APPLICATIONS OF CARBON NANOTUBES - BASED BIOMATERIALS IN BIOMEDICAL NANOTECHNOLOGY

APPLICATIONS OF CARBON NANOTUBE-BASED BIOMATERIALS IN BIOMEDICAL NANOTECHNOLOGY - REVIEW

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ABSTRACT

One of the facets of nanotechnology applications is the immense opportunities it offers for new developments in medicine and health sciences. Carbon nanotubes (CNTs) have particularly attracted attention for designing new monitoring systems for environment and living cells as well as nanosensors. Carbon nanotube-based biomaterials are also employed as support for active prosthesis or functional matrices in reparation of parts of the human body. These nanostructures are studied as molecular-level building blocks for complex and miniaturized medical devices, and as substrate for the stimulation of cellular growth. The CNTs are cylindricaly shaped with caged molecules which can act as nanoscale containers for molecular species, well required for biomolecular recognition and drug delivery systems. Endowed with very large aspect ratio, an excellent electrical conductivity and inertness along with mechanical robustness, nanotubes found enormous applications in molecular electronics and bioelectronics. The ballistic electrical behaviour of SWNTs conjugated with functionalization promotes a large variety of biosensors for individual molecules. Actuative response of CNTs is considered very promising feature for nanodevices, micro-robots and artificial muscles. An exhaustive description of CNTs based biomaterials is attempted in this review, in order to point out their enormous potential for biomedical nanotechnology and nanobiotechnology.

1.1.1 INTRODUCTION

Historically, the field of biomaterials has proven to have an outstanding potential for medical applications and has rapidly gained importance during the last decade. This development is due to the mounting demand for high-quality medical care, encouraged by the development of nanotechnologies. Indeed, new nanomaterials can lead to the creation of new supports and components for implants, artificial organs and other prosthetic devices. This increasing interest is fuelled by the fact that their use ensures accurate intervention with as little intrusion as possible and hence contributes to a very specific therapeutic effect. Owing to the small size and high contact surface area, nanomaterials possess unique potential for medical applications and thus have captured the scientist's imagination in the recent years [1,2,3,4].

One of the most intensively developing fields of nanomaterial technology is related to carbon nanostructures. Originally discovered in 1991, carbon nanotubes (CNTs), can be considered as a derivative of both carbon fibers and fullerene with molecules composed of 60 atoms of carbons arranged in particular hollow tubes [5,6]. The principal beneficiaries of nanotubes are the miniaturisation of medical devices, the development of electronic systems and the emergence of nanotools with the capacity to interact with the human body and to monitor complex interactions. For instance, miniaturization and stabilization of biosensors are facilitated by the use of robust wire materials such as

carbon nanotubes [7]. The CNTs'capacity to ensure direct electrical signals as well as readout with ultra-high sensitivity and superior response is very advantageous. Indeed, the change in nanotubes resistance as a result of chemical interactions between surface atoms and absorbed molecules can be detected in a few seconds [8,9]. These characteristics urge the use of nanotubes for the next generation of biosensors which requires the fabrication of nanotubes with well-controlled structural parameters 10,11]. The vast investigation of these unique nanostructures will permit the achievement of significant developments in bioelectronics in the last few years with a high impact in clinical medicine and biotechnology.

Owning smart behaviour, as source of the generation of an actuation force, and endowed with the exceptional mechanical and chemical stability, the CNTs reinforce new prospects for the performance of active prosthesis and artificial muscles. Identified by a high surface area, a tubular shape and the high flexibility, the carbon nanotubes possess the capacity of both reservoir and delivery systems for biomolecules. Extremely small and possessing an enormous potential for chemical functionalization, the carbon nanotube structures become a friendly support for the biological substrate and act as a very specific partner in biochemical interactions. Due to all these possibilities, the CNTs open the door for new approaches in medicine and pave the way for nanomedicine.

In this review we analyse the main characteristics of CNTs as biomaterials while focusing on the prospective applications of CNTs in medicine, in the biological field and

biomedical engineering. Starting with the introduction of nanostructures as biomaterials, this review is organized in five sections. In the second section, an overview of synthesis and nanostructural characteristics as well as intrinsic properties of carbon nanotubes will be given, in order to introduce the most important aspects of interest in the field of biomaterials. A clear understanding of structure-properties relationship is considered as well. This description regards the structural particularities, the physical, chemical and electronic characteristics along with the mechanical behaviour. Each of them is the origin of an appropriate response in different applications as is presented in section three, where functionalization of CNTs is treated as a particular way to enhance their response toward living systems. Furthermore, a short presentation of the reactivity of nanotubes in relation to their stability, dissolution and purification is done. A brief illustration of useful recognition methods is also included in the third section. The following exposition, presented in the fourth section focuses on biocompatibility, which is the main requirement of materials for medical and biological use. In the fifth one, the use of CNTs as biomaterials in different applications will be reviewed. This part summarizes some of the most important realizations in new devices such as biosensors, actuators, nanorobots, delivery system, etc. However, the purpose of this section is not an exhaustive review of all available applications and we emphasize the new avenues of medicine to which the use of nanotubes may lead. A conclusion on carbon nanotubes and their use in biomaterial field is presented in section six with a focus on challenges and opportunities.

Through this literature summary we intend to offer an overview of the CNTs' potential to add new prospects in the field of biomaterials and present the promising approaches which enhance nanotubes functionality for medicine. This review does not intend to be comprehensive, since we mainly focus on the potential of nanotubes as biomaterial and on the exploitation of their exceptional properties in new biomedical devices.

1.1.2 CARBON NANOTUBE MATERIALS

SYNTHESIS OF CARBON NANOTUBES

Several synthesis methods which allow the preparation of carbon nanotubes with different levels of purity and in a variety of structures and geometries are presented below. Special carbon nanotubes configurations and architectures have been recently reported [12] and they have prompted a lot of interest for biomedical applications.

1) *Arc-discharge technique:* This technique uses the high temperature (>3000°C) necessary for the evaporation of carbon atoms into a plasma, resulting in the formation of both multi- and single-walled CNTs. The type of gas and the pressure are determinant parameters for the nature of products; the optimal value of pressure is around 500 torr while applying a potential of 20-25 V. The presence of a catalyst is not required for MWNT, whereas the preparation of individual SWNT uses catalysts such as Co, Ni, Fe,

Y; mixed catalysts (Fe/Ni) favour the production of growth bundles of SWNT [13]. The resulting nanotubes have to be purified after synthesis with the best yield ratio of 2:1.

2) *Laser Ablation Method:* This technique allows the vaporisation of graphite in an electrical furnace heated at $1200C^{\circ}$. The graphite purity ensures a high level purity for the resulting products and a high converting ratio. SWNTs are produced as ropes with diameter between 10 and 20 nm and around 100 μm as length. The variation of parameters such as temperature and catalysts allows the variation of size [14].

3) *CVD Method:* This technique consists in the decomposition of hydrocarbure or CO under temperature $(500^{\circ}C\text{-}1200^{\circ}C)$ in the presence of $CaCO_3$, as catalyst [15]; the variation of substrates procures a great flexibility for processing.

For the biomaterial purpose, the high purity level is a concern; therefore the macroscopic processing is also employed to improve the quality of carbon nanotube materials and to obtain specific characteristics such as length, alignment, etc.

Geometric Structural Characteristics

CNTs have an unusual tubule structure which distinguishes them from any previously known carbon fibers. The uniqueness of their structure consists in the fact that each CNT is a single molecule wherein each atom has an identified conformation. From the structural point of view, CNTs are usually described as an arrangement of carbon hexagons that form tiny tubes; they can be regarded as a true macromolecular system with the architecture of ideal graphene sheets [3]. A single wall carbon nanotube is a

tubular form of carbon with diameter ranging from 0.4 nm to 2-3 nm and a length that varies from a few nanometres to several microns. The majority of multi-wall carbon nanotubes consists of rolled graphite layers which are either folded in one another, or wrapped around a common axis with an interlayer spacing of 0.34 - 0.36 nm; the inside diameter is 0.4nm and the outside diameter is about 5nm [16,17]. The existence of certain topological defects in the structure leads to the formation of curved structures [18]. In these arrangements, the nanotube ends with a hemispherical cap including regular pentagons in its structure along with the usual hexagons [19,20]. The presence of pentagons at the tube's ends suggests that the nanotubes should be considered as a limiting case of the fullerene molecule, whose longitudinal axis length considerably exceeds its diameter [21].

CNTs form in two fashions: single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs), which were the first to be discovered. The so-called single-walled carbon nanotube (SWNTs) [22] is the closest to an ideal fullerene fiber and consists of a single graphene sheet wrapped up in the form a tube [21,22]. These single layer cylinders, with diameters varying between 0,4 and 2 nm, extend from end to end and aggregate into bundles. They are organized into larger ropes that consist of several tens of nanotubes assembled in one dimensional lattice with a lattice constant of 1.7 nm and a tube distance of 0.315 nm. The MWNTs are made of concentric graphitic cylinders placed around a common central hollow. They can be distinguished from single walled ones because they adopt considerably more configurations and shapes with

larger variation in diameter, from 1, 5 nm to 100 nm [5,16]. In fact, the MWNTs are close to hollow graphite fibers, except for their tendency to have a higher degree of structural perfection [16,17].

Since a CNT exists as a rolled-up graphene sheet, the bonding mechanism in the carbon nanotube system is similar to that of graphite, and thus, characterized by sp^2 hybridization [18,21]. All carbon atoms have four valence electrons, three of which form sp^2-hybridized σ bonds to the neighbouring atoms giving graphene high plane rigidity. The fourth electron (π-orbital) is delocalised and shared by all atoms forming the conduction band, thus allowing for electronic current transport. The rolling-up is done in a helical fashion with respect to the tube axis; this feature, known as *helicity*, provides structure for an individual nanotube and creates a fascinating potential for the engineering of electronic properties [22,23]. It was proven that CNTs are exceptionally good conductors: the SWNT is a ballistic conductor whereas for MWNT this issue is more complex because of the additional electronic coupling between adjacent shells [24,25].

Among the various ways of defining the unique carbon nanostructure, the most employed is the one based on the *unit cell*, which groups the smallest number of atoms [17]. It is characterised by the **chiral vector** defined as: $C_h = n\hat{a}_1 = m\hat{a}_2$, where $\hat{a}_1$ and $\hat{a}_2$ are unit vectors in the two dimensional lattice, and *m, n* are integers which determine the value of tubule diameter and chiral angle. Each nanotube topology is related to these integers (n, m) which define a particular symmetry. The C_h vector connects two

cristalografically equivalent sites on a 2D graphene sheet with a *chiral angle, θ*, which is the angle it makes with respect to the zig-zag direction [17]. Depending on the orientation of the graphene layers, with respect to the nanotube axis, three major categories of SWNTs can be defined: i) the *armchair*, in which n = m and the chiral angle is 30°; ii) the *zig-zag* form corresponding to n = 0, and $\theta = 0$; iii) *chiral* nanotubes are all other nanotubes with chiral angles intermediate between 0 and 30. In these arrangements the orientation angle (θ) is very important because it determines the chirality of the nanotube, and governs its electronic properties; different orientations lead to different electronic properties [17,26].

The diameter is simply the length of the chiral vector divided by 4 and plays a significant role in determining electronic properties. Indeed, based on this theoretical prediction, in 1998, experimental measurements demonstrated that the nanotube behaviour, either as a metal or as a semiconductor, strongly depends on its diameter and its chirality [23]. For instance, all armchair nanotubes are metallic, and the zig-zag can either be conductor or semiconductor. In general, a nanotube will be metallic if the relation $|n-m| = 3q$ holds true and a semiconductor otherwise. This relation is a consequence of the electronic structure of the graphene sheet, which is a semiconductor with zero band gap. There is a strong correlation between nanotube topology and its electronic structure which gives rise to distinct features; the preference in formation of one of these categories is explained in terms of cell unit of a carbon nanotube [24, 27].

In fact, the structure of multilayer nanotubes greatly depends on the production methods [12, 18] and its variability is manifested in both longitudinal and transverse directions [16]. Since the chirality is specific for each shell of multi-walled structure, the electronic properties are defined for each of the layers. Moreover, there are interactions between the shells compounding the same multi walled carbon nanotubes.

CNTs Properties

A number of parameters influence CNTs properties. As presented above, the curvature of nanotube and its local topology play a significant role in its characteristics. The presence of various impurities such as catalyst particles remaining from the fabrication process, could affect the structure. In addition, several defects relevant to rehybridisation, incomplete bonding and topology often appear on the side wall as well as at the open ends [28,29]. These defects become starting points for the development of noncovalent and covalent chemistry of nanotutubes [30] in multiple directions. However, it was proved that CNTs can tolerate only a limited number of defects before macroscopic samples lose their special electronic and mechanical properties.

Electric Properties and Electronic Structure

The remarkable electronic properties of carbon nanotubes offer an immense potential for novel application in both the biotechnological and the medical field. The ability of SWNTs to display fundamentally distinct properties, without changing the local

bonding, sets nanotubes apart from all other nanowire materials [23,31,32]. Depending on the method of preparation CNTs can either be insulators, semiconductors, or conductors. The understanding of the conducting properties of carbon nanotubes is related to their electronic structure. In spite of their simple chemical composition and atomic bonding configuration, the structure-properties relationship is quite strong.

The conductance of nanotubes: First predicted in 1992 [33], the electronic properties of CNTs, were further elucidated by experimental measurements and observations [34]. It was demonstrated that the electrical properties of nanotubes extensively depend on specific parameters (m,n) and therefore on diameter and chirality [35]. The investigation of semiconducting and metallic SWNT clearly confirms the remarkable electronic behaviour of nanotubes that may function as moderate gap semiconductor [36,37]. This behaviour plays a major role in the construction of the tip probes and sensors [38] which hold a myriad of promises for the new generation of biosensors [39,40].

Emission characteristics of nanotubes: The electron field emission of SWNT was observed at an electric field strength exceeding 16 Vmm^{-1} while for MWNT it is of higher magnitude. The maximum value of the field emission current density corresponds to 3A cm^{-1} and is attainable for both types. These results support the electron work function for the film surface (1eV), thus recommending nanotubes as the best material for electron guns [41] dedicated to the development of field emission transistors (FET). Such a system, endowed with conducting channels, offers an alternative for the detection of binding proteins and endorses the construction of devices for protein identification

[42,43]. Moreover, using both non covalent and covalent side-wall chemistry, with the effect on bulk separation of tubes, the development of specific interactions between molecules and materials has been achieved [44] with great impact on the selective functionalization in molecular electronics, including field-effect transistors.

Magnetic properties

A specific characteristic of electric conductivity of nanotubes consists in its pronounced dependence on the magnetic field. It was predicted that the presence of a magnetic field will strongly affect the band structure of carbon nanotubes near their fermi [45]. Indeed, a rise in conductivity as a function of the magnetic field [46] was demonstrated by several experimental works. The change in this character was also confirmed when doping the material with metal atoms. Measurements of magnetic suscebility, as an indicator of the magnetic performance, confirmed the diamagnetic properties of nanotube bundles. Though not completely elucidated, this particular behaviour found application in the medical field, especially in the MRI, in delivery systems and target therapy [47].

PHYSICAL PROPERTIES

As structures combining both molecular and solid state properties, the CNTs could be considered as an intermediate state of a substance [35], with multiple inferences.

Specific Surface: Many applications of CNTs are based on their high specific area, feature allowing to use CNTs as porous materials. Indeed, the capacity of nanotubes to shape the oriented spiral-like structures leads to the formation of a large number of nanometer-sized cavities attainable openings for penetration of gas and liquids from the exterior. For nanotubes material the value comes close to that of an individual nanotube, and ranges over a very broad scale, from several dozens to several hundreds m^2/g; the value for SWNTs is about 600 to 1000 m^2g^{-1} [35]. This value increases with purification degree and the highest one, experimentally reported was of 1587 m^2g^{-1} corresponding to HiPCO with specific treatments. The construction of electrodes for high capacity and high specific electrochemical capacitors takes advantage of this feature. Moreover, the bio-adsorptive properties of nanotubes surface efficiently influence the biotechnological processes. For instance, the preparation of nanofilters used for separation of nanometer-size virus or bacteria, such *Escherichia coli*, holds a major advantage [48]. These robust, nanoporous filters allow a reproducible filtration process; more tailoring is achieved by controlling the nanotubes density in the walls or by surface chemical functionalization. The immunomagnetic separation of *E. coli*, in pure and mixed cultures, was tested by the application of new systems including albumin functionalized MWNTs with encapsulated ferromagnetic elements conjugated with pathogen-specific antibody [49]. These developments offer new approaches for separation techniques valuable in biotechnology.

MECHANICAL BEHAVIOUR

CNTs mechanical properties greatly exceed those of any previously known material. High Young modulus (E), stiffness and flexibility have been demonstrated through theoretical modeling and confirmed by experimental studies [50,51,48]. The nanotube's modulus is a measure of its stiffness against small axial stretching and compression strain, as well as non-axial-bending and torsion strains on the nanotubes. An average value of 1 800 GPa [51] was determined for E, but a more realistic one of 1 200 GPa, has been reported for SWNTs. The results show that the elastic properties, the strength and rigidity of nanotubes largely depend on the distribution of defects and geometric features [53]. Though the strength and stiffness should be comparable to that of graphene sheet, in the case of tubular shape, there is a relationship between the elastic strain energies and the intrinsic curvature of C-C bonds [54,55]. Studies carried out at room temperature prove that, under stress, the tube typically yields 5-10% axial strain [55], with a tensile strength around of 50 GPa [56,57]. The high flexibility of nanotubes is ascribed to their ability to rehybridize sp^2-sp^3; the higher the curvature, the more dominant is the sp^3 character in the C-C bond in the deformed regions. In fact, the nanotubes recover from severe structural distortion [19] and can thus sustain an extreme strain (40%) without showing any plastic deformation, signs of brittleness or bond rupture. Moreover, by adapted macroscopic process [58], the nanotubes can form continuous, infinitely long ropes, resulting in a significant improvement of the stiffness of the nanostructures.

The world of these structures hides attractive phenomena [16]; their exploration by appropriate avenues, using high resolution techniques, sustains the identification of new suitable functions. Advanced probe microscopy techniques such as atomic force microscope (AFM) and Scanning tunnelling microscope (STM) has offered new opportunities for the nanoscale study of mechanical behaviour of CNTs [59,60].

CHEMICAL CHARACTERISTICS

The understanding of CNTs chemistry is crucial for their development as biomaterials and their use in biomedical applications, with particular interest for biochemistry [61]. As in the case of fullerene, the reactivity of CNTs raises from their topology and non planar carbon atoms. The fullerene chemistry is definitely determined by pyramidalization angle whereas in the case of nanotubes the misalignement angle and tube diameter also have an important contribution and differentiate them during the reaction. For instance, SWNTs are characterised by strong covalent bonding, a unique dimensional structure and nanometer size which imparts unusual properties to the nanotubes; a perfect SWNT has no functional groups and is hence chemically inert [62].

There is a strong relationship between the electronic structure of fullerenes and their chemical characteristics. Unlike the planar form characterised by a trigonal, sp^2 hybridization of carbon atom with a $\theta_p = 0°$, in C_{60} all carbon atoms have $\theta_p = 11.6°$, more

appropriate for sp^3, tetrahedral hybridisation (θ_p=19.5°). This configuration favours the addition chemistry [63] explained by a permanent susceptibility for chemical conversion from trivalent to tetravalent carbon which relieves the strain at points of attachment and saturates the carbon atoms. This reactivity directly depends on the curvature; its increase leads to a more pronounced pyramidalization of the sp^3 hybridized carbon, thus increasing its tendency to undergo an addition reaction, especially with very reactive species [64].

From a chemical point of view, a nanotube is divided into two regions: the end caps, with θ_p= 11.6° and side-wall with θ_p= 6.0°. This difference is generated by curvatures: the end caps, curved in 2D, are similar to those of hemispherical fullerene, whereas the 1D curved side wall nanotube contains less distorted carbon atoms. Due to their specific curvatures, the caps seem to be much more reactive than the nanotube walls which are considered to be inert and exclusively require highly reactive agents for covalent functionalization. Accordingly, the reactivity of SWNTs is relatively lower than that of flat graphene [63].

Oxidation: Chemical oxidation techniques were used to prepare highly functionalised nanotubes containing acid groups [64]. The first results are related to the oxidation of CNTs in the gaseous phase [19] and show more reactivity for nanotube tips than for the tube itself. This effect is more present in solutions where treatment with strong acids lead to the apparition of functional groups such as carboxylic acids (– COOH). Contrary to the tip which, due to the location of pentagon defects at the tube's

end, is easily subjected to oxidation, the structure of the cylindrical surface generally displays a resistance to oxidation [19,65]. The tip geometry is another key parameter enhancing the oxidation rate by its possibility to generate higher stress at the ends. The reaction usually starts in the outer layer and progresses inward, resulting in the attachment of many functional groups (–OH, –COOH, –CO) on the surface [66]. This change in reactivity facilitates a better bonding and further modifies the wetting characteristics, as presented in section III.

1.1.3 REACTIVITY AND FUNCTIONALIZATION

CNTs Reactivity

The reactivity of nanotubes and consequently their stability are absolutely decisive for their biomaterial functions. Using chemistry not only enables the purification of the pristine or the dispersion of nanotubes bundle into individual ones, but it also promotes the creation of new functions for biomaterials. In this paper we consider two aspects of the nanotube reactivity. The first is related to CNT inertness and regards the response of nanotube when in contact with the living body. This stability greatly contributes to their biocompatibility, consisting in the capacity of nanotubes to be accepted by a living system. The second aspect considers the fabrication process and the modification approaches which should be a possible source of alteration of the basic chemical stability. Indeed, the evidence that mechanical deformations of nanotubes such as

bending, twisting or flattering greatly influence their electronic properties, even acting as preferential sites of molecular absorption, [67] has been proved. As a result, the chemical response could be modified as a function of the applied deformations. Several other defects [30,68] influence the reactivity of SWNTs and they can serve as an anchor group for dissolution and functionalization [69]. It was recognized that a large proportion of the defect sites, particularly those located on side-walls, become useful for activation of nanotube surface with various polymers, amides or esters [29,70,71,72]. Hence, bimolecular species can be attached, in various ways, along the CNT to form hybrid assemblies with new properties. Furthermore, using the chemical modification of the nanotube surface new structural properties are attainable in nanocomposites polymers [73] incorporating carbon nanotubes, resulting in improvement of mechanical behaviour.

CNTs FUNCTIONALIZATION

CNTs seem to be the ideal support for miniaturized implanted devices because of their small size, chemical inertness along with their unique electronic and mechanical properties. However, the ever-growing applications of nanotubes in biology and medicine can be hindered by the difficulty to integrate such nanostructures into biological systems. In this context the lack of solubility of nanotubes in aqueous conditions is a concern [30,74].

One way to overcome this limit is the CNT functionalization which creates new functions thus favouring the coupling of nanotube characteristics with those of other materials such as biologic molecules or functional polymers [75], rendering them more amenable for integrated systems. This avenue ensures the development of new materials [76,77] and encourages the development of supramolecular systems for molecular actuators [79] and molecular electronic applications [77,78]. Moreover, surface functionalization is essential in producing advanced materials with good bulk and desirable surface specificity for biosensors and probes. Thus, nanotube tips and sidewall modification have been reported with significant results for covalent or noncovalent chemistry [79,80,81]. While the former is exploited to create chemically sensitive proximal probe tips, the second one is a versatile way to induce surface specific interactions. For instance, the integration of nanotube structure in a biological assembly enables the creation of a complex architecture in organic systems [82,83,84,85]. It is evident that CNTs functionalization involves both molecular and supramolecular chemistry, following various approaches. They include covalent and noncovalent modes [30] concerning the endohedral or exoehedral parts as well as the sites generated by the presence of defects.

i) Covalent functionalization

The covalent chemistry of carbon nanotube is a promising method for the design of new biomaterials. Initially used for CNT solubilisation, the covalent chemistry has become a forerunner to many biological applications such as nanotube tips endowed with biological and chemical discrimination capacity. By using acidic functionality and by

coupling basic or hydrophobic functionalities or biomolecular probes to the carboxyl groups, molecular probes have been created [30,86]. Polymer and dendrimers with amino and hydroxyl groups can be attached to nanotubes in order to obtain amides and esters derivates [30]. As presented herein, the nanotubes participate to addition reactions in different ways according to their topological and geometric parameters. Creation of covalent, non-polar C-C bonds on the walls results in the breaking of the local sp^2 hybridization and the formation of π-π conjugated bond at surface [87]. However, the modification of mechanical behaviour and alteration of electronic structure are the undesirable consequences of these changes. Studies based on molecular dynamic simulations predict a decrease of 15% of the maximum buckling force by covalent attachments due to the introduction of sp^3 hybridized carbon defects [88]. There are different covalent approaches to chemically modify the CNTs and their versatility is described below.

Oxidation: The first experiments of successful chemistry of nanotubes have involved treatment of nanotubes with sulphuric and nitric acid under oxidative conditions. This reaction was used as a cutting procedure, opening ends, as well as a way to introduce oxygenated functionalities such as carboxylic acids, quinones and esters. The ozone treatment is also used to introduce such functionalities [30,71,79] and their exploitation has taken many forms. For instance, the creation of carboxylic acid which favours the access of peptides via amide linkage [71] is useful for biological applications. The dependence of reactivity on curvature strain encouraging the rapid oxidation of thinner

nanotubes [71,89,90] was also exploited; it has been pointed out that this type of modification is more specific for the caps area.

Reduction: The direct sidewall functionalization of SWNTs using a reduction workout can be achieved by hydrogenation or by using reactive species such as nitrene [70], carbenes [90] or aryl radicals [78,91].

Carbene and radicals: Generally, the degree of functionalization of the resulting products varies and greatly depends on the diameter of nanotubes and on the method used for fabrication of nanotubes [85, 86]; a wide variety of nanotubes derivatives is produced.

Fluorination: Initial attempts to functionalize sidewalls nanotubes were using the fluorination reaction [86]. The side wall modification was done for the first time in 1998 [87] employing the buckypapers and elemental fluorine. Thus, a fluorine covalently bond was formed resulting in drastically change of electrical conductivity of new material. This change is a consequence of rehybridization of carbon atoms, which take place rather around the circumference of the tube than in the direction of its axis [94]; the results are in agreement with the theory of the addition mechanism around of the circumference of the tube. The results of fluorinated SWNTs can be employed for the solubilisation of carbon nanotubes and will reveal new opportunities for sidewall functionalization [95] with the application in integration of nanotubes in functional nanostructures.

Aryl diazonium reaction: This method enables the preparation of functionalized SWNTs and involves a buckypaper electrode in its reaction with aryl diazonium

compound. The use of the covalent chemistry gives rise to amine- SWNTs which were further covalently linked to DNA [96]. This multi-step route enables the formation of highly stable DNA-SWNT products, possessing complementary sequences with minimal interaction. They can be used as building blocks for more complex supramolecular structures as well as in highly selective, reversible sensors. Accordingly, DNA hybridization provides a potential pathway to manage complex systems by taking advantage of the high degree of selectivity and reversibility as well as of the ability to readily design, synthesizes and link different DNA sequences to a variety of surfaces [97]. Hence we can significantly modify the chemical behaviour of nanotubes and adapt them to a specific biological environment or to particular medical functions. The complexation of functionalized SWNT with nanochitosan contributed to the improvement of delivery capacity resulting in the design of new delivery system for peptide and DNA with enhanced characteristics compared to chitosan alone [98]. The major inconvenient of covalent reactions is that it could alter the inherent properties of nanotubes [99] as presented above. In spite of this inadequacy, the method leads to derivatives of nanotubes, favourable to the preparation of material with variable side-wall functionalities [100] and for cutting nanotubes.

ii) Non-Covalent functionalization

The nanotube surface chemistry becomes a critical aspect in chemical applications because every atom is on the surface. For instance, self assembly on the surface and preparation of biosensors is based on side functionalization. In order to maintain the sp^2 nanotube structure and thus, its electronic characteristics, the noncovalent pathway is

preferred. The preservation of extended π-networks of nanotubes is realized through chemical absorption. Hence, the supramolecular assemblies on the CNT surface have been obtained by using sodium dodecyl-sulphate (SDS) at micelar concentration and different lipids after dialysis of the surfactant [101]. It seems that the presence of surfactant in a concentration higher than the critical micelle concentration (CMC) is very important. The absorption of the hydrophobic part of the surfactant can be reversed when the concentration of surfactant is below the CMC. In the case of using proteins, the lipidic chain appears to be a crucial factor in the formation of these assemblies, whose stability is controlled by thermodynamic factors. Such arrangements are exploited for the development of new biosensors and bioelectronics using both types of nanotubes [71].

The absorbing capacity of CNTs for SDS, resulting in homogenous dispersion, found its application also in macroscopic processing of CNTs materials [102] with successful results in the orientation of carbon nanotubes in the field of a laminar flow consisting of polymer.

iii) Functionalization and Immobilization of Proteins: One of the most important applications of functionalized CNTs is related to the immobilisation of biomolecules on a nanotube in order to obtain a biosensor substrate. Indeed, the capacity of biomolecules to be absorbed on MWNT via hydrophobic interactions between the nanotubes and hydrophobic segments of proteins [103] has been proved. The method developed by the Dai group [104] employs a bifonctional molecule, 1-pyreno-butanoic acid, succinimidyl ester which is irreversibly absorbed on the hydrophobic surface of SWNT in an organic

solvent. The nucleophilic substitution and formation of amide bonds enables the immobilisation of a wide range of biomolecules with high efficiency and specificity. The extension of this approach to small molecules or polymerisable compounds opens the possibility of self-assembly of nanotubes without perturbation of their sp^2 structure.

The construction of stable supramolecular assemblies using functionalized nanotubes and lipidic chains has previously been presented [101]; the water insoluble double-chain lipids were found to be organized at the nanotube surface in a similar way with the mixed micelles of SDS, resulting in stable assemblies. According to the symmetry and the helicity of carbon nanotubes in single or multi-wall form, the shaping of supramolecular structure can be guided. In addition, the investigation of absorption of metalloproteins and enzymes on the nanotube's surface [105] sustains that a variety of enzymes could be immobilized on the tubes, with detectable retention of activity. The nanotubes-proteins interactions engage non-specific participation of proteins along with the contribution of covalent and electrostatic forces, thus resulting in a robust immobilisation of nanotubes. These results demonstrate the ability of nanotubes to absorb a variety of bimolecular species, on both internal and external surface, while maintaining their intrinsic properties. This behaviour is useful for practical applications in the development of bioelectrochemistry. It was shown that the assemblies of amino groups onto nanotube sidewalls covalently link phosphate groups. Thus, the thionine-MWNT modification found application in the construction of new electrochemical biosensors with improvement of the detection limits [106].

PURIFICATION, DISSOLUTION AND WETTABILITY

The synthesized nanotubes are always accompanied by other species [107,108], independently of the method used for their fabrication. Consequently, the purification is required to achieve the separation and removal of catalyst particles, support material and amorphous carbon from CNTs [109,110]. Highly purified CNTs are generally required for biomedical devices, since the amount of impurity prevents this kind of application for two reasons. First of all, their intrinsic characteristics become weaker affecting their biofunctionality and secondly, once in contact with the living body, they could cause secondary effects which finally affect biocompatibility. It is thus essential to ensure a high purity level of CNTs in order to use them as biomaterial. This process can be facilitated by dissolution, with effects on soluble impurities [104,107,111]. As presented herein, specific functions of CNTs in the biomedical area are achievable through chemical functionalization [95,112,113]. However, prior to functionalization, it is convenient to remove all sources of contamination [114] with the most successful results in organic solvents. Besides, most of the research groups achieved both solubilisation and functionalization of carbon nanotubes using the same pathway. One of the most employed methods for SWNT purification is the treatment with nitric acid. In fact, it is possible to oxidise the nanotubes with a variety of agents: oxygen, carbon dioxide and treatment with oxidizing acids [107] followed by a gas phase oxidation process. The most widespread purification methods are based on the attack of oxidizing agents such as HCl, HNO_3, capable of efficiently removing metal particles and eliminating

carbonaceous impurities [112,113]. The combinatory action of high temperature and hydrogen treatment ensures [114] the high quality of products. The preparation of CNTs based biomaterials strongly depends on their dissolution and on their purity level, which undeniably requires nanotubes in aqueous media. One of the main roles of nanotube dissolution consist in obtaining a high state of dispersion while preserving their structure. Moreover, the deficiency related to nanotube solubility in water [102,103], can be overcome by using covalent and noncovalent chemistry [97,101, 104].

Strategic approaches toward the solubilisation of CNTs involving chemical and physical modification have been developed [115] with applications in biochemistry and medical sciences. Hence, the solubilization of SWNTs in starch aqueous solution was performed using starch-iodine complex [116]. The results evidenced the contribution of enzymatic hydrolysis to the integration process of SWNTs with other biosystems. A new process based on molecularly controlled encapsulation of CNTs using helical amylose turned out to be a simple, fast and efficient tool for dissolution of nanotubes in water [117,119].

It is known that capillarity is a prerequisite for wettability with further improvement in processability and biocompatibility. The modification of the carbon configuration of nanotubes changes their polarity and thus, the wetting of nanotube becomes closest to planar graphite [94]. It was found that wetting is no longer obtained for a liquid with surface tension higher than 200 μNm^{-1}; this means that low surface tension liquids such as organic solvents wet nanotubes. Furthermore, capillarity forces are used not only to

fill nanotubes with small molecules but also to coat nanotubes externally and uniformly [95].

1.1.4 BIOCOMPATIBILITY OF CARBON NANOTUBES

Biocompatible behaviour is imperative for a successful function of implantable devices once introduced into the body. The intrusion of a nanomaterial into the body triggers substrate effects at the nanoscale level at which structural components of biological systems are built, thus encouraging a strong affinity between molecules. In spite of some limitations related to the processing, the dissolution and the purity level, the variety of nanotube applications in medical and biological areas is in continuous increase, thus the need to further study biocompatibility issues. Being insoluble in organic solvents and aqueous media, CNTs display the tendency to aggregate and form a non uniform dispersion. In this respect, chemical modification of carbon nanotubes has been demonstrated to be the best method to engineer these materials. Indeed, such adaptation is really helpful to eliminate this technical barrier [119] since the functional groups attached to the surface readily react with chemical reagents and further guarantee a homogenous dispersion. Beyond this useful application, the modified nanotubes behave as a material whose biocompatibility must be proven, despite the known capacity of the living body to integrate carbonaceous materials [120].

In the light of these trends, the biocompatibility of nanotubes becomes more and more current issue in relation to the research which explores and exploits these materials for medical use. In fact, in the place where the inserted nanostructured material takes seat in the body, a response arising from the interactions between the surface and tissue appears at local and systemic levels, thus determining the biocompatibility. One can thus speak about the concept of biocompatibility in the traditional sense, when referring to medical implants. However, the effect of specific interactions at nanoscale [121] grounds new issues regarding the biocompatibility of nanomaterials. More specifically, these relations are related to the use of new structures in the construction of miniaturized medical devices, such as micro- and nano-robots [120]. In this prospect, biocompatibility studies of nanostructured materials should be investigated at two levels: i) from a traditional point of view, by approaching all the effects relatedto the interactions engaged by device implantation; ii) the biocompatibility of nanomedical materials. While in the first case there is much knowledge about carbon biocompatibility, in the second approach, the investigation of nanostructures and nanomaterials is still under development [121]. Indeed, the discovery of nanotubes launched a new paradigm in the domain of biomaterials. It started in 1998 when the question «could carbon nanotubes be toxic » was addressed for the first time [122], thus taking into account the impact of nanotubes on health. Furthermore, in 2001, the results of the first assessments on the biocompatibility of pure fullerenes [123] showed no association with any health risk. The authors rather believe that the major problem is especially related to the inhalation of these forms, attempting to understand the mechanisms underlying these effects. These

tubes, with a diameter of 1 nm and length of a few microns, are compared with asbestos, « like fibers asbestos», which is related to cancer [122,a]. However, Mossman, pathologist and expert of asbestos, doubts that nanotubes can have similar behaviour, supporting that the cancerogeneicity of asbestos is rather correlated to its capacity to generate reactive compounds [122,b] than to its structure. In spite of their geometric similitude with asbestos, it seems that the size & shape matter is opportune only in the case of inhalation of nanotubes. Thanks to their stability, the nanotubes cannot be broken up quickly by the cells and hence, persist a longer time. Therefore, the carbon structure does not react in the same manner with the cellular components to engender poisonous by-products. If this assumption holds true, the mechanisms inducing the toxic effects are not known. Recent works have shown that nanotube cytotoxicity is partially caused by the presence of residual metal catalysts as well as the insolubility of this material; this statement not only strengthens the role of purification but it also sustains the purpose of nanotubes functionalization [124]. Furthermore, the derivatized SWNTs can initiate the attachment of small molecule such proteins; the resulting compounds are particularly important for protein and gene delivery applications endorsed with dose-response ability. In fact, the understanding of hydrophobic-hydrophilic balance of carbon nanotubes in relation with synthesis and post-processing is very important. A proper comprehension of hydrophobic interactions facilitates the regulation of protein adsorption, thus tailoring the surface of nanofibers for their use in biomedical applications [125].

The first studies regarding CNTs [126] toxicity considered carbon nanotube fibres instilled in the body and the formation of glaucoma has been reported. This inherent inflammation effect is probably due to the electrostatic nature of the nanotube and not to its individuality. At the same time, Lam and collaborators [127] reported pulmonary toxicity for three types of carbon nanotubes; although produced by three different methods, using various metal catalysts, their toxic effects were similar.

Still under exploration, the biocompatibility of SWNTs has been studied using HEK293 cells as research target, in view to investigate the effects of nanotubes on these cells and to explore the biochemical mechanisms [128]. These observations demonstrated the influence of SWNTs on the proliferation of HEK293 cells. Reversely, the cells can induce an active response, such as secretion of small proteins, to isolate cells attached to nanotube from the remaining cell mass; these events could initiate a pathway for disease therapy. Moreover, it appears that the interactions between nanotubes and cells are a priority, greatly depending on the fabrication and preparation of the material, including functionalization [129]. Thus, aiming to explain the cause of dermal irritation in humans after exposure to carbon fibers, a complex MWNT structure, not designed for biological applications, has been tested. The human epidermal keratinocytes were exposed to various concentrations of MWNT solutions, resulting in an irritating response in a target epithelial cell. In spite of their evidence, these results are not able to explain neither the mechanisms nor the effects. Therefore, functionalized SWNTs were analysed for degree of functionalization, dispersion in water and cytotoxic response in mitochondrial activity

[130]. As a result, significant interactions have been distinguished, demonstrating the ability of nanotubes to induce the bio- nano interface with beneficial effects for development of system delivery or diagnostic devices. On the other hand, a biocompatibility study of highly purified SWNTs in contact with cardiomyocite in culture [131] suggests that the long-term negative effects can be induced more by the physical parameters than by chemical interactions; no short-term toxicity has been evidenced.

Taking into consideration the chemical stability of carbon nanotubes, the bioactivity has to be studied in direct relation to their biocompatibility. Recent works focused on various specific activities of nanotubes and demonstrated the capacity of single walled nanotubes to activate the human monocytes and the mouse splenocytes to produce TNF-alpha [132]. A new combination including carbon nanotubes and Fe_2O_3 has strong effects on the inhibition of pathogenic bacteria growth in water. This photocatalytic killing activity of bacterial cells finds application in the purification of drinking water from pathogenic bacteria [133]. Another specific activity toward biologic cells was detected in relation to pH environment [134]. It was observed that DNA wrapped HiPCO carbon nanotubes, consisting in a stable aqueous dispersion, possess a unique optical pH response with great interest for application in optical biosensors.

The results presented herein strengthen the tremendous necessity to systematically investigate the relation of carbon nanotubes with various cells, in multiple conditions, in order to understand the biocompatibility of carbon nanotubes as well as their specific

activities. This theme still remains a subject that raiseslines multiple questions related to the toxicity profile of carbon nanotubes, the efficiency of their derivatives as well as the adaptability of CNT-containing nanodevices.

1.1.5 BIOMEDICAL APPLICATIONS OF CARBON NANOTUBES

ARTIFICIAL MUSCLES AND ACTUATORS

An artificial muscle analogous to a biological muscle is composed of an actuator material that must be able to work in specific ways in order to successfully mimic the natural one. The natural muscles are distinguished by some characteristics: anisotropic behaviour consisting in contraction-elongation along the fiber axis, high energy density, fast speed and response, as well as large stroke. With a maximum power output of 150-225 W/Kg, they can withstand a stress up to 150-300 KPa. The work of muscles is defined by three variables: the stress which they can exert, the strain by which they can be shortened and the contraction frequency [135]. Actuation capacity consists in the ability to efficiently convert electrical energy into mechanical movement and its requirements are defined in terms of stroke and force. Generally, an actuator is composed of at least three elements: two electrodes whose function is to apply the potential, and an interposed element [136]. The ideal actuating material would operate at low voltage and at a matchable performance of skeletal muscle [137]: 10% strain, 0,3% MPa stress generated and 10% s^{-1} strain rate. Characteristics such as light, low-volume,

long cycle-life, large displacement and high forces are required for actuator design and some of them correspond to CNTs [138].

The nanotube's actuation behaviour is generated by its good electronic and mechanical properties coupled with a high surface area as well as a non-faradaic nature of the electrochemical response. Owing to these properties, the nanotube actuators perform high strain per movement and generate greater mechanical stress than any other material, [139] when a voltage of a few volts is applied. In this type of actuator, the nanotube acts as an active material and constitutes one of the electrodes which are immersed in an electrolytic solution or solid polymer electrolyte [138,139]. Theoretical predictions and experimental measurements have suggested that actuators using carbon nanotubes allow high performances consisting in higher work densities by cycle [138] and have significant contribution in term of time and speed as well as reproducibility of its response.

CNTS ACTUATORS FOR ARTIFICIAL MUSCLES

In 1999 [138] Baughman has demonstrated, for the first time, that carbon nanotube can act as an actuator thus proposing the first prototype for a bimorph actuator. The macroscopic sheets, so called buckypapers, consisting of randomly entangled SWNT bundles act like working electrodes, connected to a potentiostat in electrochemical cells. An axial strain of up to 0,2% was provided by using bukypaper while switching the electrochemical potential from 0,5V to -1,0 V. A roughly parabolic dependence on sheet

dimension, with a minimum length corresponding to 0,5 V was observed. In fact, the mechanism for converting electrical energy into mechanical energy is based on the double-layer charge injection and it involves the interface nanotubes-solution [137,139]. The actuation is due to a geometrical expansion of the C-C covalent bonds caused by charge injection and originates from a quantum chemical and double layer electrostatic effect [140]. Such response, devoid of ionic contribution, eliminates the disadvantages related to the faradaic conducting polymer actuators, for which the life cycle, the discharge rate and the energy conversion efficiency are limiting parameters [141]. This new mechanism is sustained by the structural model of SWNT, its electrical conductivity along with mechanical and charge transfer properties [138]. Following this assertion, the non-faradaic actuation concept has been predicted by theoretical studies and experimentally demonstrated using both individual and ropes SWNTs as well as the MWNTs [140]. The first isometric measurements shows high values for elastic modulus, of 1-2 GPa, and maximum deformation of 0,2%, corresponding to a stress equal to 0,75 MPa. In fact, individual nanotubes display much better mechanical and electronic properties, expressed by a stress of 1 000 GPa and 1% actuation strain. It has been demonstrated that the structural deformation as well as the conversion of electrical energy into mechanical force, through radial and longitudinal expansion or contraction, are more due to the electronic structure than to the coulomb interactions. Since then, several works were performed attempting to explain the actuation performance of nanotubes and hence improve these characteristics [141]. They principally focus on both the fabrication and manipulation of CNTs in order to obtain suspended nanotubes [140].

By manipulating these nanotubes with AFM tip, and examining the change in cantilever deflection, the measurements of both the Young's modulus and actuation force are easily performed [140,142,143,144]. Moreover, Frayse and his collaborators [144] studied nanotubes obtained by CVD growth and deposited on trenched quartz substrate with the NT's ends fixed. They thus estimated the actuation capability under bias voltage in an electrochemical cell by measuring the deformation of nanotubes with AFM cantilever [140]. According to the hypothesis that a limited macroscopic response arises from the building and entanglement of individual nanotubes at meso-and macroscopic scale, the authors found the possibility of taking measurements at individual nanotube level. In the same prospect, Roth and Baughman experimented with individual nanotubes as actuators and demonstrated that the nano-objects change their shape when electrically charged [145].

Since the purification level of nanotubes was identified as a concern for the efficiency of the actuation process, cumulated efforts contributed to the development of highly purified, aligned CNTs, with a high surface area [112,113,114]. Moreover, significant improvement of actuation strain rate was achieved by operating carbon nanotubes in an organic electrolyte [146,147]. For instance, the use of resistance compensation resulted in a higher rate, of 0,6% s^{-1} [148] with an increasing pulse amplitude and more negative potential limit. Thus, a strain amplitude of 0,3% in 0,5 s was attained which is considerably better than 0,5% in 1 s, previously reported. Despite a small displacement of nanotubes, these results sustain the undeniable actuation capacity of advanced carbon nanotube structures and forecast their promising potential [149] for actuators with a

performance similar, in some respects, to that of a biological muscle. This CNTs resource to emulate artificial muscles opens the door for nanoscale devices [150] with various applications. Other actuating designs, such as implants for tissue stimulation [151,152] or electrical stimulators capable of inducing ostoeogenesis are envisaged [153]. In combination with biopolymers, such as collagen, [154], or functionalized, [155] carbon nanotubes are processed as material for the creation of engineered tissue or as components for other diseases. Although our focus is not the presentation of carbon nanotubes as macroscopic material, we mention that the advantages coming from this dirction are very enriching for the design of biocompatible biomaterials. Indeed it has been proven that the insertion of functionalized CNTs in a collagen scaffold induce positive effects [155] in terms of physiology, electrical conductivity and mechanical behaviour.

With regard to the macroscopic formulation of the CNTs, it is important to note that composite structure is another alternative of material which could improve actuator performance. In this context SWNT-Nafion composite actuators were created and tested [156]. The results qualify the system as promising potential for the construction of MEMS switches. Moreover, combinatory actuator effect of both carbon nanotubes and conductor polymer, such as polyaniline, are in exploration [157]. Film composites were tested in order to evaluate the actuation synergism of two components in a new actuator material. The influence of electrolyte has also been evaluated by using both salt and basic solutions. Thus, the contribution of a redox process was evidenced while

demonstrating the increase in conductivity of the composite by participation of CNTs, leading to enhanced electrochemical efficiency. It is more evident that material processing offers a diversification of smart materials for actuators. Indeed, the experimental work on nanotube-epoxy layered actuator is promising for the development of dry nanocomposites actuator material [158]. In spite of the manufacturing challenge, it appears that these composites may become a new smart material for structural application, which could promote the development of dry actuator, as component of active prostheses.

BIOMOLECULE AND CARBON NANOTUBE ASSEMBLIES

The first step towards the synthesis of peptide based carbon nanotubes started a few years ago with the achievement of derivatives of SWNTs and MWNTs with n-protected aminoacids [118]. The method is based on the addition reactions on nanotube external surfaces and its solubilisation in aqueous media. At the same time, an exhaustive work on solubilization of SWNT has been directed towards the assembly of coated peptides [119].

Nanotube based supramolecular structures have been prepared by controlling the factors that influence peptide-peptide interactions [159]. Based on both molecular modeling and experimental data, an amphiphilic α-helical peptide model was developed with the aim to predict the self assembly function. This construction is the first step towards a new architectural arrangement of carbon nanotubes for molecular sensing mechanisms. Thus,

a recent identification of polypeptides with selectivity for nanotubes [160] announces a new method for the manipulation and use of CNTs in the biological and medical area. These peptide sequences, with specific affinity for nanotubes, naturally bind to the surface of carbon nanotubes, in a selective manner; this ability is owed to the flexibility and conformation of their chains. It seems that peptidic chains act as symmetric detergents, with hydrophobic sequence in the middle and hydrophilic ones at the ends. Their inherent selectivity enables them to discriminate between different nanotube structures, according to their chirality and diameter, and thus facilitates the CNTs manipulation for biosensors.

The multifunctionalization of CNTs finds application in the design of systems for delivery of antibiotics to different types of cells. Using selective transport through the membrane, a new approach is proposed for the modulation of therapeutic actions [161]. Such a strategy was experimented in the last year and demonstrated, *in vitro*, the aptitude of oxidized MWNTs to conjugate with both fluorescin and amphotericin (AmB) molecules. Even though AmB is known to be the most effective antibiotic for cronical fungal infection, a high toxicity risk appears when in contact with mammalian cells. Consequently, the advantage of this new conjugated system consists in its capacity to partially avoid the aggregation of molecules resulting in increased aqueous solubility for AmB and internalisation capacity of CNTs, which finally improve antibiotic activity.

The influence of interfacing of carbon nanotubes with biomolecules is various [162] and could be extended to certain reactions which they influence. Indeed, an investigation of

effects generated by addition of SWNT to the polymerase chain reaction [163] illustrates an increase in efficiency of polymerase mechanism, proving the capacity of nanotubes to act as catalysts in a variety of biochemical reactions as electron/donor receptors. With regards to biologic cells, carbon nanotubes showed excellent conditions for the proliferation of cell culture [164]. These effects can be useful for therapeutic application of carbon nanotubes in theregenerative processes.

CNTs NEURAL BIOMATERIAL

Neural prostheses provide the means to apply and monitor electrical signals in neural tissues. The development of systems able to restore nerve function does not strictly lie in the realm of neuroscience, medicine and engineering; new possibilities arise at the interface of all three and demand the use of advanced biomaterials. Carbon nanotubes possess excellent electrical properties which have been proven useful in the area of neural prostheses. The pertinence of nanotube fibers as neural prosthetic biomaterial has been demonstrated by McKenzie and his collaborators [165]. Thanks to their capacity to minimize the function of astrocites, the CNTs possessing high surface energy become interesting biomaterials for a new generation of neural prosthesis. Haddon and his colleagues reported the application of carbon nanotubes in neural research and established the function of nanotubes as a support for nerve cell growth as well as substrates for probes with neuronal function at nanometer scale [166]. Using nanotubes of diameters similar to those of small nerve fibers, they developed methods for growing

embryonic rat-brain neurons on MWNT. The chemically modified MWNT coated with 4-hydroxynonenal bioactive molecules stimulated neurite growth with extensive branching. A similarity between the diameter of nanotubes, ranging from 1 nm for SWNT and 10-100 nm for MWNT, and those of neurites, favours localized molecular interactions, necessary for the formation of a neuronal circuit. Furthermore, the conductivity of nanotubes renders them a valuable candidate for electrophysiological analysis of neuronal micro circuitry. It is known that neural development is based on a complex phenomenon that requires, among others, the outgrowth of neurites [167]. The investigation of CNTs as neural biomaterials requires biocompatibility studies. Investigating the *in vitro* cytocompatibility of CNTs [168], the authors demonstrated the beneficial effects of carbon nanofibers of limiting astrocyte functions, leading to decreased glial scar tissue formation and further establishinf a relationship between fiber characteristics and astrocyte interactions. These investigations promote the development of miniaturized devices in which the nanotubes play a role in the formation of a nerve cell network with an active function, possibly to contribute to the restoration of a damaged neuronal circuit. These encouraging results recommend CNTs as promising neural biomaterial for the innovation of implant devices for the central nervous system. Today this area is limited by the use of silicon material which shows the capacity to induce significant glial scar tissue formation [169] which is known as a common difficulty in the field of neural prosthetics. Indeed very recent results demonstrating the biocompatibility of native and functionalized single-walled carbon nanotubes for neural applications [170] promote the CNTs' potential for regenerative processes.

CNTs FOR THE DELIVERY SYSTEMS

Three characteristics of nanostructured materials recommend them for the design delivery system: i) nanoscale size enables intravenous injection; ii) small particle size minimizes possible irritant reactions at the injection site; iii) miniaturised size allows the penetration of carriers through the membrane of the sick cells, thus providing a way to selectively deliver the drug to cancerous tumours, while avoiding the healthy cells. As presented in the section about functionalization, functionalized nanotubes and their derivatives are destined for biomedical and biotechnological applications. Indeed, the organic modification of nanotubes creates multiple attachment sites for bioactive molecules and hence facilitates the assembly in complex nanodevices. A very recent study demonstrated the capacity of nanotubes to serve as vehicles for the administration of vaccines [84,85] by developing new and effective delivery approaches of protective antigens. They offer the possibility to effectively use the antigens while enhancing and modulating the immune response. The potential of CNTs to present a biologically important epitope, with the appropriate conformation, *in vitro* and *in vivo* tests, enables them to be used in vaccine delivery. The peptide-carbon nanotube conjugated system, integrating aminoderivatized nanotubes selectively bonded to a peptide, provides the peptide bis-adducts [85]. This conjugated system elicited strong anti-peptide antibody response and retains the antigen conformation, which fulfills the main requirements for the induction of an antibody response, with the accurate specificity. Since nanotubes do

not have any detectable immunogenicity, the system is very appropriate for the delivery of a vaccine antigen. More specifically, new structures are designed using water soluble SWNT derivatives which are able to cross the cell membrane [84]; they can be accumulated in the cytoplasm or reach the nucleus without being toxic up to a quantity/concentration of 10pm; their investigation as delivery systems in targeting therapy [171] demonstrated that the absence of immunogenicity of nanotubes increases the efficacy of their function.

MINIATURIZED DEVICES AND NANOROBOTICS FOR NANOMEDICINE

CNTs ELECTRODES AND BIOSENSORS

CNT Electrode: Carbon nanotube is a better electrode material than the classical carbon and its development becomes a relatively new topic in electrochemical studies. The useful electrochemical properties combined with its high surface area provide some advantages to the field. Although the CNTs inertness is evident, it has been proven that the chemical environment to which the nanotubes are exposed influences their conducting capacity and thus creates an additional transduction mechanism. It has been demonstrated that CNTs can enhance the electron transfer when used as an electrode in electrochemical reactions [9,11]. An important advantage lies in the fact that these materials are very sensitive to various molecules with the reactivity controlled by the redox system. Moreover, due to their highly specific capacitance, the CNTs can be used

as electrochemical devices. The well-aligned MWNTs based electrodes have demonstrated their successful application for the detection of uric acid in urine samples with excellent sensitivity and selectivity [172]. When employed as an electrode, the relationship between structure-topology and properties enables the use of the enzyme-immobilized CNTs for biomolecular sensing or for a miniaturised sensor in gas environmental analysis. These chemically modified CNTs enhance the electrode sensitivity and selectivity [173]. Therefore, using layer-by-layer methodology, the nanoscale biosensors were constructed. These electrodes allow generating an electrochemical signal as function of substrate concentration, resulting in very good detection limits [174].

CNTs Biosensors: One of the most important and far reaching application of nanotubes is in the field of biosensors where their biorecognition function is combined with signal transduction. Combining biological selectivity with the electronic process, the biosensors provide direct information about the chemical composition of their biological environment. They constantly screen the presence, absence or concentration of organic or inorganic substances with a rapid time of response, thus allowing a continuous real time monitoring of analysts. The biosensors not only have a stand–alone identity but they also act as part of medical devices for the detection of toxic substances [175,176] or therapeutic drug monitoring [177,178]. Several biological events such as genotoxicity, immunotoxicity, biotoxin and endocrine effects are measured using biosensors [179,180,181,182].

The sensing process is divided into two parts: recognition, which results in selectivity and amplification which increases the power of weak signals to a level at which it can be conveniently manipulated by electronic devices. The multiplicity in transduction mechanisms and amplification, processing variety along with its suppleness in elements recognition create a wide range of biosensors. Five principal groups of biologically sensitive materials can be used for selective recognition: i) enzymes; ii) antibodies; iii) nucleic acid; iii) receptors and iv) intact cells. These macromolecular biological compounds are properly attached to the transducer surface through immobilization processes which do not only enable the fixation of the receptor to the surface but also improve the stabilisation of biological materials. Research efforts pursued in the last few years mainly focus on the development of new techniques involving bio-nanosensors effective at the single cell level, where the detection mechanisms involve chemical interactions between nanotube surface atoms and the absorbed molecules [119,175].

CNTs provide an excellent platform for biosensors and even for integrated systems, such as arrays, able to analyse the chemical and biological environment. Owing to their high surface area and outstanding electrical properties, the nanotubes performance is superior when compared to conventional materials. Experimental results clearly demonstrate that electrical conductivity changes as the carbon nanotubes are exposed to a minuscule amount of a certain gas molecules [10,16]. Thus, SWNT based chemical sensors [183] display a dramatic change in their electrical resistance when exposed to gaseous

molecules such as NO_2, O_2 and NH_3, [184,185,186]. Recent achievements have confirmed the aptitude of individual SWNTs to detect the smallest concentrations of toxic gas molecules, such as NO_2 and NH_3, as documented by Dai and his group [187]. In fact, this molecular wire benefits from the full exposure of nanotube surface to chemical environment, thus conducting to a fast response and sensitivity.

Moreover, the capacity of CNTs to detect ppm-level compounds at room temperature allows the development of nanotube sensors at physiological temperatures because, once fully immersed in water, the SWNT still maintains its intrinsic electronic properties.

Such ability propels nanotubes towards the design of miniaturized implantable devices such as, biomedical nanosensors with high impact on nanomedecine [187,188,189]; these nanodevices will further promote innovation in early detection diagnostic techniques. More specifically, the sensitive detection of nanotubes-based probes in conjunction with the modulation of mechanical or electrical characteristics favours the interactions with a biologic host, i.e., the deposited cellular materials on the surface [190,191,192].

Endowed with a high specificity, these devices are dedicated to accurate control of environment [193,194,195] and even for *in vivo* and *in vitro* evaluation [196,197,198]. Thus, a drastical change in electrical properties of nanotubes, in response to the surrounding environment, is converted in highly specific, sensitive signals as predicted by theoretical studies [199] and demonstrated by experimental measurements [200,201].

In fact, the potential of nanotube as electrodes for *in vitro* and in *vivo* investigation was experimented, for the first time, in 1996 when the Ajayan's group constructed a nanotube -electrode for neurotransmitters involving dopamine, using bromophorm as binder [198]. The voltamograme profile, corresponding to the oxidation of active molecules, such dopamine, has demonstrated the superiority of the CNT electrode, versus the untreated one. This new attainment of the electrode has been attributed to the carbon nanotubes size, as well as to their electronic structure combined with their topological parameters.

Moreover, in 1998, the foremost demonstration of the CNTs potential for protein immobilisation [202] has proven that small proteins are not only immobilised at the nanotube surface, but they can also be readily placed within the interior cavity of the opened nanotube. This assertion reveals the capability of SWNTs to act as a benign host for the selective encapsulation of proteins inside the tube, which is a real potential for biosensors. At the same time, Gao has investigated the possibility of deposing DNA molecules on robust nanotubes, thus proving the nanotubes capacity to recognize different biomolecules as a function of their diameter and helicity [203]. These progresses contributed to a better comprehension of the interactions between nanotubes and molecular species, in addition to their sensing mechanisms. Furthermore, a relationship between electronic properties and the absorption of gas molecules was formulated [204].

Theoretical calculations indicate that the molecule absorption on the surface or inside the nanotube bundle is stronger than that on an individual tube. In addition, the

impressive properties of nanotubes, such as nanometer size, large surface area, and chirality - dependent on electrical conductivity, have been exploited for building the nano- arrays, functioning as biosensors or double layer capacitor, with higher resolution. Hence, the use MWNT arrays as an immobilization matrix for the development of amperometric biosensor have been reported by Sotiropoulu and Chaniotakis [10]. Using oxidation for opening and functionalizing of the nanotube array leads to an efficient immobilisation of glucose oxidaze enzyme. In this system, a platinum substrate acts as direct transduction platform for signal monitoring, whereas the nanotubes play a dual role, as an immobilisation matrice as well as a mediator. The reported results are very promising for the third generation of biosensors. The SWNT capability to act as a building block for nanobiosensors, dedicated to the sub-cellular kinetic studies, has been investigated by Guisepi-Elie and collaborators [205]. It seems that the nanotubes can perform as a benign host enables to trap and support biological molecules while the acidic sites present at the surface enhance molecule binding. This resulting proximity facilitates the redox process of proteins and enzymes from a kinetic and energetic point of view. Thus, coupling the SWNTs with redox active enzymes favours nanobiosensors development, i.e., the evolution of glucose biosensors [206,207,208,209,210]. This functional enrichment, resulting from nanoscale surface modification of implantable devices, triggers many surface interactions with biological host tissue, in order to either evade the host's immune system or to successfully engage it in a mutual reaction [211].

Noncovalent functionalization of SWNTs [212] has also been considered for the development of highly specific electronic biomolecule detectors, with a selective recognition, enable to target proteins by the conjugation of their specific receptors [195]. These biosensors easily work in the clinical detection of biomolecules, such as antibodies, associated with human autoimmune diseases. Two principal applications have been found for this array system: the first brings into play the selective detection of proteins in solution and considers the detecting serum protein after a vaccination or a therapeutic intervention. The second one involves the use of purely electrical transducers in proteomic applications with the aim to detect different proteins without labelling them.

On the other hand, the nanotubes compound with a polymer results in the production of composite material for the construction of electrochemiluminescence-sensing device [211]. Adapted for immobilization of streptavidin, they are attractive for the realization of a nanotube based quantitative biosensor, capable for wide range measurements of biological analytes, with application in areas of medical diagnosis, drug discovery, etc. Moreover, the construction of a 3D electrode for a stable, very sensitive and high selective glucose sensor was realised by using aligned CNT coated with conducting polymer favouring enzymes accessibility, thus increasing efficiency [172]. The use of CNTs facilitates the development of single enzyme biosensors, with activity toward thiocoline, without the use of mediating redox species. A surface modification of MWNT electrode makes possible immobilization of the enzyme [213] in a simple construction which performs with good precision and excellent limit of detection whilst

ensuring good stability and reproducibility. Therefore, significant advancements are attempted in clinical medicine, enabling direct electrical detection of biological and chemical agents. In fact the preparation of polymeric nanocomposite from nanotubes and PVA [214] and CNTs / nickel hexacyanoferrate nanocomposite [215] resulted in significantly improved electrical conductivity and electro catalytic activity resulting in good electrochemical performance for glucose detection when used as an electrode. Moreover, using the bio-composite principle, a CNT epoxy composite biosensor was built which offers an excellent sensitivity and stable electrochemical properties combined with a reliable calibration profile [216].

CNTs FLOW SENSORS

The excellent sensing capacity of nanotubes was extended to the flow conditions resulting in systems for flow environment. Gosh and his collaborators reported the experimental observation of the voltage generated by the flow of a polar liquid over SWNT bundles. The magnitude of voltage induced along the nanotube significantly depends on the ionic strength and polar nature of the liquid [217]. A high sensitivity along with a fast response time was detected at low velocities, following the direction of the current versus the fluid flow. These results demonstrate the ability of nanotubes to act as flow sensors for small volumes, in a flowing liquid environment, which is ideal for biomedical applications.

CNTs NANOSENSORS AND PROBES

Carbon nanotubes are considered the primarily building materials for nanosensors because they function both as sensor elements and as electrical contacts [173]. An innovative, alternative method for the fabrication of nanowire sensor arrays was developed using an electrodeposition technique. The main advantages are related to the effectiveness of this process to produce individually or addressable nanowire sensor arrays with multi-chemical sensing capabilities, thus stimulating the evolution of carbon nanotube based nanosensors [218]. Such versed sensors are capable of fitting inside single cells thus capturing information related to the concentration of proteins in living cells during the body's normal operation; they play a major role in the development of miniaturized, implantable devices for full-time detection of medical problems. Hence, the construction of nanotube based probes offers new possibilities for the sensitive detection of single particles and single molecules [219]. The SWNTs are very hopeful for conducting probe techniques as AFM or STM, endowed with high lateral resolution, high aspect ratio and good resistance. Through functionalization, the nanotube becomes an ideal tip for biological molecule probes [220,221] and opens up the possibility of inserting a nanoscale electrode into the small pore structure. These devices have the capacity to measure the length dependence of electrical transport [222].

Interfacing nanotechnology with biology enables the integration of carbon into highly aligned and water stable process resulting in electrically-conductive probes [223]. With

several hundred microns in length, the CNT probes have the ability to easily penetrate the cell membrane of epithelial cells. Such local probes operate in a manner which preserves cells viability; this is a new promising technique for nanoassembly of nano-devices and nano- tools. Moreover, new instruments for performing cell-level surgical operation has been performed by using AFM and fabricated probes shaped as ultrathin needles [224]. This technique enables the extended of AFM principle to analyses and surgery of living cells. In fact the needle enables to penetrate the cell membrane and could be easily insert into a nucleus with highly accurate positioning. Furthermore, recent theoretical studies demonstrated the possibility to obtain the arrangements as DNA-CNT arrays which could perform as functioning device [225]. This complex system can be used as an electronic switch or as device for DNA sequencing.

Announcing a promising function for CNT as neuron-probes, the recent results reveal the CNTs capacity to offer a high spatial resolution required for probing neurons [15]. Endowed with high electrical conductance, the small and flexible nanotube enables the detection of the electrical signal from neuron to neuron. Since the concept of nanotube bio-nanoprobe has been proven, several studies have been performed in order to evaluate the detection and discrimination capacities of CNT probes towards patterned samples, using molecular interactions and forces in bimolecular interactions [222]. Thus, the use of nanotubes as probes has gained great interest in the recent years. A review paper covering the most important aspects related to this application has been presented [226].

Topics regarding structural and mechanical characteristics, fabrication methods along with tip characteristics, resolution as well as functional imaging, are featured. The tips obtained by different methods [227], provide images of biomolecules or bio-assemblies, with a very high lateral resolution owing to nanotubes robustness [222]. New triple-probe atomic force microscope, consisting in an AFM system coupled to carbon nanotube nanotweezers, was tested for the first time with the aim to measure the electric properties of a tri-terminal single DNA molecule device [228]. Hence, the operation of a single DNA molecule as a three-terminal device has been demonstrated, leading to new opportunities for DNA electronics. Moreover, currently works demonstrated that changes in electronic characteristics of carbon nanotube FET can be correlated with DNA detection, demonstrating that DNA adosorption and hybridization were selective for nanotubes [229]. This design, with certain modifications, could serve as nanosensor for discrimination of DNA in a milliliter of blood.

CNTs FET DEVICES

Field Effect Transistors (FETs) have been fabricated by using semi conducting single wall nanotubes. Their extensive study confirmed that they are extremely sensitive to the chemical or biological environment (oxygen) surrounding them [174]. The capacity of proteins to crystallise in a helical configuration on the nanotube surface [104] favours the topological compatibility between nanotubes and conformation of organic products, such as proteins. On the other hand, the carboxylic open ends of nanotubes, possessing

an important hydrophilic behaviour, are not attractive for hydrophobic proteins; they thus permit a specific reaction with analyte, leading to its identification [219].

A new technique for the electronic detection of proteins was recently experimented using CNTs as a conducting channel. Taking advantage of the capacity of biotin-streptavidin to bind to CNTs, the authors produced a specific architecture that uses the nanotube field effect transducer and polymer functionalization to obtain a supramolecular assembly [45]. The application of a conducting polymer coating leads to the conversion of nanotube from p- to n-type conductor, thus preventing non-specific attachment of proteins. However, the coating with a hydrophilic polymer results in a reduction of the affinity of nanotube for proteins. Such modifications enhance the activity of a miniaturized device, acting as a rapid sensor within individually detect proteins or virus.

The recent works reported a selective reaction pathway of SWNTs, in which chemical functionalization is controlled by the difference in the nanotube's electronic structure [220]. The authors anticipate the extension of this concept to cell-based electronic sensing devices for the measurement of electronic responses to living systems, with *in vivo* applications. For instance, the construction of a complex architecture coupling SWNT to peptide nucleic acid (PNA) was performed [221]. This structure intermediates the attachment to DNA, by hybridization at room temperature. Moreover, the incorporation of SWNTs into larger devices by interfacing nanotube ends with PAN enables the creation of highly selective nanotube sensors, offering a new approach for

complex devices, with selective recognition of target proteins in conjugation with their specific receptors.

CNTs CATHODE FOR IMAGING X-RAY RADIATION

The high performance CNT-based field emission cathode for the design of x-ray tubes has been reported [230] by using CNT film with optimized morphology. This device readily produces both a continuous and pulsed x-rays in a programmable wave form and with a finite repetition rate. The tubes are constructed using purified SWNT bundles created by laser ablation; they produce focused electron beams with small energy spread which is potentially used as x-ray energy tubes, for high resolution imaging with ultrafine focal spots. This CNT cathode x-ray technology can potentially lead to portable miniature x-ray sources for specific medical applications. Recent works [231]. presented the development of a dynamic radiography system with CNT based microfocus x-ray tube that can generate pulsed x-ray radiation with a temporal resolution as short as 50 ns, significantly better than thermionic x-ray tubes In spite of some limitations which could be overcome, this compact and versatile system is promising for non-invasive imaging devices for biomedical research, as proven by the first results [231].

CNTs NANOTWEEZERS FOR BIOLOGICAL APPLICATIONS

The nanotweezers, a powerful tool for manipulation and performing electric measurements at nanoscale level, fully exploit the actuation effect of single nanotubes.

Their construction is based on individually connected, free-standing carbon nanotubes sitting on two metal terminals separated by a 50 nm gap [232]. The application of a voltage to two electrodes causes an electrostatic attraction of the nanotubes, thus closing the two ends. Such nanoscale electromechanical system was realised, for the first time in 1999 by Kim and Lieber [233]; the electrically conducing and mechanically robust nanotubes were attached to independent electrodes made of pulled glass micropipettes. The free ends of nanotubes alternatively open and close, while applying a voltage to the electrode. These nanotweezers enable the manipulation of individual nanostructures, and their electrical properties can be harnessed in several manners [233]. One of them finds application in the biological field, for manipulations/modifications within a cell. The construction of dual mechanical/chemical nanotweezers is based on the self assembly principle, through bio-recognition on the molecular level [232]. The resulting tubes have 50 nm in diameter and quite uniform length, with the possibility of being quantitative control by the time of exposure. Functionalized nanotubes are tested for new applications in cell immobilization and transport measurement [232] able to ensure biological recognition.

CNTs - NANOPIPETTES

The nanopipettes, as new shaping of nanotubes, are based on a new morphological manifestation of carbon nanostructure, characterised by a hollow conical structure. This 1-3 nm hollowness remains constant throughout the length of the structure [234], while

the shape grow up, thanks to the simultaneous growth of nanotubes during the chemical vapour deposition process, with the atypical composition for gas phase. These whiskers with some minor faceting on the surface have a pointed tip, in the shape of pipette, and the base at submicron size. These new carbon nanotube pipettes could be ideal candidates for simultaneous drug delivery and in vivo detection of neurotransmitters [234].

CNTs - COMPONENTS FOR MEDICAL NANOROBOTS

Nanorobotics is concerned with the interaction between atomic- and molecular-sized objects and mainly deals with their controlled manipulation [235]. Nanorobots are nanodevices which, in a futuristic vision, will be used for maintaining the human body and protecting it against pathogenic agents. Having a diameter of about 0.5 to 3 microns, nanorobots could be constructed out of parts with dimensions in the range of 1 to 100 nanometers. They will allow surgeons to perform direct in vivo surgery on individual human cells [235]; the powering of nanorobots can be supplied by metabolising local glucose and oxygen for energy, or external energy. In spite of the complexity requirements for the design and development of nanorobots, theoretical and simulation studies are on going. This first step aims to explain the role of nanorobot behaviour and to further propose new approaches and strategies [236] for future developments. In the light of properties and functions presented herein, it becomes visible that carbon nanotubes are a very promising support for some nanorobots components [237].

Generally, the objectives of nanomanipulation refer to the 3D manoeuvring of chemical compounds for molecule building which will be further assembled into larger devices. Thus, in living bodies, nanomanipulation involves nano-scale biological entities such as DNA and proteins for the clinical and scientific analysis. This requires materials for the fabrication of nano-devices or components for integrated systems, with very specific properties. Indeed, by manipulating carbon nanotubes at both the atomic and molecular scale, the biological assemblies integrating carbon nanotubes are more accessible [235].

On the other side, nanorobots could assist the immune system by finding and disabling unwanted bacteria or viruses. When an invader is identified it can be punctured, letting its content spill out and thus ending its efficiency [238]. By following a predetermined search pattern, the nanorobots can ingest and destroy harmful bacteria they encounter by using mechanical and chemical phagocytosis. Such devices enable to distinguish every cell type by checking their surface antigen by chemotactic sensors keyed to specific antigens target cells. Nanorobots would even work in the bloodstream where they could nibble away at arterisclerotic deposits, widening the diseased blood vessels [239, 240].

Carbon nanotubes and their nanocomposites offer support for nanoelectromechanical systems (NEMS) and micro- and nano-robots; endowed with high strength and chemical inertness they can thus avoid an attack by the host's immune system. Recently, new methods have been developed in order to construct, at the nanometer scale, building

blocks of carbon nanotubes with these desired features. These 3D assemblies, built with great accuracy, could be functionalized using biological specimens. Indeed, based on controlled nanostructure interactions and complexity, nanoassemblies can posses a higher density and thus become better electrical conductors, faster circuits as with more complex functions and with reduced power consummation [241]. Such sophisticated architectures are in reality quite similar to biological entities with nanoscale organization. This resemblance facilitates the system's integration to the living body, resulting in the establishment of much more natural interactions, thus offering a greater control and high resolution monitoring of the phenomena, without secondary effects [235, 240]. Moreover, building a high level programming system for their manipulation is a big challenge since it involves the integration of various components [241].

In fact, the design of CNTs based SPM is currently in progress and will play an important role in the advancement of nanorobotics [242]. Some representative development's elements are: i) substrate that serves as nanofixture; ii) tips, probes and molecules that serve as grippers or end-effectors; iii) chemical and physical nanoassembly processes; iv) primal nanoasssembly operation for insertion; v) methods exploiting selfassembly thus eliminating spatial uncertainty; vi) adequate hardware for building nanostructure; vii) algorithms and software for sensory interpretation; viii) motion planning; and ix) SPM driving. In response, CNTs obviously exert a multiplicity of functions [243] in nanoelectronics, NEMS, and other nanodevices.

Even though the fabrication of a complete circuit at the molecular level remains a challenge, the realisation of CNT based nano electronic devices is presently in progress and hence, paves the way to nanorobot production. As presented in a previous section, a complex system has been manufactured using CNT probe tip [189]. This system is the precursor of tips for molecular resolution imaging and in perspective, a constitutive element for nanorobots [221].

1.1.6 TRENDS FOR THE FUTURE: CHALLENGES AND OPORTUNITIES

The CNTs contribution to biomaterials appears to be fascinating and they are of a major significance for the medicine and biomedical applications. However, the scientists' preoccupation concerning the potential risk of nanostructures to human health raises an uncertainty. Interpretation of information regarding the toxicological effects generated by inhalation and handling of carbon nanotubes suggest that a policy of industrial hygiene measures must be elaborated. Additional confusions relating to the effects of their surface area and geometric parameters are pulling alarm trigger about the potential risks and therefore, necessitate precise requirements for the use of nanotubes into the body. Therefore, a sensible research able to answer the growing concern regarding unintended negative impacts would be of a great value for the enormous interest generated by the use of carbon nanotubes in engineered nano-biomaterials with advanced functions for biomedical applications.

From molecular-scale components of nanoelectronic devices to nanoactuators and nanorobots, the nanotubes will be the support of a revolutionizing class of devices with diagnostic and therapeutic aims. Moreover, the in-progress nanoscale research enhances nanotechnology's contribution to medicine and thus new prototype devices will be able to overcome most of the limits of modern medicine. Theoretical models and experimental research demonstrated there bewildering potential. The chemical inertness of CNTs along with their nanoscale dimensions renders them perfect candidates for biological and biotechnological applications [84,85,171]. Their intrinsic properties enable their bioconjugation for biosensing applications [16,187,188]. Moreover, the remarkable combination of electronic and mechanical properties of CNTs makes them a potential building block of revolutionary electronic devices and nano-electromechanical systems with applications in the biological and biomedical fields [137,144, 232, 234, 240].

As biocompatible support for biological assemblies or nanomechanical part in integrated systems, these new materials become components for implants, artificial organs and other prosthetic devices [153,156,157]. They contribute to the advancement of medical applications and promote a new approach to nanomedecine, with safer, more efficient and ultimately, more powerful tools. Biosensors offer a wide range of new biotechnological enhancements; they are the key to a number of major projects in medical technology such as the development of artificial organs that mimic the function of real organs more accurately. Biosensoring technology, based on carbon nanotubes

will be unique due to its high sensitivity, selectivity, stability, size, and mobility [175,176,178,211,212,221].

Device miniaturization and the creation of robots for nanomedecine will be promoted by new designs and sophisticated models [239] incorporating carbon nanotubes. Functionalized CNTs allow the construction of new devices for the identification of specific binding sites and chemically distinct domains of proteins [54,71]. These nanotube based devices offer new opportunities for the detection of biological species clinically, as well as important sensing interactions with a well defined transfer function [188,228]. Many new effects allow the creation of materials and machines with unique nanoscale properties without the macroscopic counterparts [9]. Another fascinating application is molecular recognition involving both classes of biomolecules, antibodies and nucleotides [203,221,171].

We conclude on the marvellous role that carbon nanotubes could play in the development of biomaterials in the future, while emphasizing on the challenges related to their fabrication and biocompatibility. Based on ongoing progresses, more concretizations are expected in their formulation at macroscopic level, in sheets, fibers or composite forms. Solving this problem will enable to extend the exploration of the unique properties of nanotubes, confirmed at the individual level, and will instigate original initiatives in the promotion of new biomaterials.

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1.2 POLYLACTIC-CO-GLYCOLIC ACID (PLGA)

1.2.1 *Polylactic-co-glycolic acid (PLGA) Copolymers*

The polylactic-co-glycolic acid, PLGA, is a synthetic copolymer of two poly(α-hydroxy acids): the poly lactic acid (α-hydroxy propanoic acid) and poly glycolic acid (hydroxy acetic acid).

$$\left[\begin{array}{c} O \\ \| \\ CH-C-O \\ | \\ CH_3 \end{array}\right]\left[\begin{array}{c} O \\ \| \\ CH_2-C-O \\ 2 \quad 1 \end{array}\right]$$
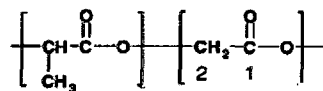
PLGA

Both are synthetic biodegradable polyesters with particular characteristics of interest for biomedical applications. Lactic acid contains an asymmetric carbon atom, and therefore has two optical isomers: l(+) lactic acid and d(-) lactic acid. Lactic acid is present in nature as either an intermediate or as end product in carbohydrate metabolism; It is widely distributed in all living systems (man, animals, plants, and microorganisms). Glycolic acid occurs in nature to a limited extent. PLGA copolymers are approved by the U.S. Food and Drug Administration (FDA), and araise a significant interest for medical applications because they are less toxic, biocompatible and biodegradable. Additionally, poly(lactide-*co*-glycolide) degrades *in vivo* into innocuous products, the lactic acid and glycolic acid, which can be eliminated from the body through the tricarboxylic acid cycle as carbon dioxide and water [1] PLGA copolymers are

widely used in various medical and pharmaceutical applications due to their biodegradable and non-toxic character [2]. Biocompatible and possessing adjustable properties and process abilities, the PLGA finds extensive biomedical utilisation, such as sutures, orthopaedic fixation devices, and drug delivery systems. An advantage of the lactide/glycolide copolymers is the well recognized resourcefulness in polymer properties consisting of comonomer ratio, molar mass, polymer crystallinity and the corresponding performance characteristics, such as rate of degradation [3]. The glycolide: lactide ratio along with the molecular weight plays a part in controlling the size and morphology of micro- and macro-domains that copolymer form after dilution in organic solvents and aqueous suspensions. Several works demonstrated that 50:50 ratio allows the formation of a smaller domain versus the other combination. These characteristics play an important role in the formulation of nanoparticles and micospheres and the control of their degradation rate [4] .

1.2.2 PLGA Biodegradation

Biodegradable polymers are highly desirable components for scaffold design, since their disappearance over time will improve porous scaffold characteristic. PLGA is a biodegradable polyester with bioresorbable characteristics useful as support for sutures, fracture fixation devices and drug delivery systems. The great tailorability of PLGA biodegradation is an asset, especially when combined with non biodegaradable components such as carbon nanotubes. Being synthesized from poly(lactic acid) (PLA)

and poly(glycolic acid) (PGA), the PLGA eroding characteristics vary with the ratio between both segments. It has been demonstrated that copolymers with shorter lactide repeat units degraded faster than ones with longer lactide blocks. This response is related to the surface erosion mechanism; increasing the lactide chain length should increase hydrophobicity and hence diminish water uptake [4]. PGA is highly crystalline, as it lacks methyl side groups, and the ratio PGA:PLA determines the cristallinity of the copolymer. PLA is more hydrophobic than PGA and the ratio PLA:PGA will influence the hydrophilicity of the copolymer: the higher the lactide ratio, the slower the rate of biodegradation. A larger hydrophobic lactide copolymer could also lead to stronger hydrophobic interactions. Degradation of PLGA occurs by hydrolysis at a rate strongly dependent on the ratio and the molecular weight. The degradation time can vary from a few weeks to many months. It has been reported that PLGA containing (PLA:PGA) 50:50 ratio is hydrolysed much more rapidly than PLGA containing a higher proportion of the two monomers. Other parameters influencing the rate of copolymer biodegradation are: molecular weight and environmental factors, such pH, temperature, aqueous medium. PLGA copolymers are amorphous materials whose Tg strongly depends on molecular weight and increases with the lactide/glycolide ratio. PLGA undergoes in vitro degradation in aqueous environment by means of cleavage of their backbone ester linkages. This is a bulk process following three main steps: i) Penetration of water into the polymer: in this incubation step there is no a change of molecular weight; ii) Induction, consisting of an increasing hydration and decrease of molecular weight determining a saturation decrease; iii) Polymer erosion, consisting of total weight

loss and change in the rate of chain scission. It is an auto-acid catalytically accelerated degradation process due to the presence of carboxylic acid end groups generated in the interior of the polymer chain network, producing a more intense process in its interior than on the surface.

1.2.3 Nanoprecipitation of PLGA

The development of PLGA nano and microparticles has progressively emerged with interest in the development of systems for controlled release of active agents. This concept uses the properties of colloids in several ways and implies the dissolution of copolymer followed by its solidification. For PLGA copolymer dissolution, the use of an organic solvent is in relation to the non-toxicity of the solvent, such as acetone. Acetone is a satisfactory solvent for low and middle molecular weight products; it is freely miscible with water in all proportions. Moreover, it has been demonstrated that upon mixing an organic phase containing acetone and PLGA with an aqueous phase, the PLGA immediately precipitates. Indeed, the copolymer is transformed in nanosized domain (aggregate) without surfactant. Acetone is removed by evaporation and the composition behaves as an aqueous suspension.

Many approaches are used for the preparation of PLGA nanoparticles. Emulsification-evaporation, spontaneous emulsification-solvent diffusion and nanoprecipitation are all commonly used methods for preparing various diameters of PLGA nanoparticles. Formation of nanoparticles by emulsification-evaporation is assisted by a toxic organic

solvent such as CH_2Cl_2 and $CHCl_3$. To meet the requirements for clinical use, the residual solvents should be completely removed from PLGA particles. The aggregation of PLGA nanoparticles during the solvent-evaporation process is a critical aspect. In order to prevent PLGA nanoparticle aggregation, polymer stabilizers, such as poly(vinyl alcohol) (PVA), poly(vinyl pyrrolidone, are often used. These stabilizers cover the surface of PLGA nanoparticles and can affect particle size and surface properties[5]. Although polymer stabilizers may prevent the aggregation of nanoparticles, they are difficultly removed even by washing. The nanoprecipitation method is useful for preparing pseudolatexes. The latter are derived from colloidal polymer dispersions after removal of organic solvent, used to dissolve the preformed polymer. They differ from common latex prepared from water insoluble monomers by emulsion or dispersion polymerization methods. The major advantage of pseudolatexes lies in the feasibility of the conversion of these polymers into an organic colloidal dispersion, allowing direct incorporation of plasticizers. When a biocompatible polymer has to be dissolved, preferentially non-toxic organic solvents are used. In particular, for the biocompatible copolymer such as PLGA, acetone is largely employed. When using a nanoprecipitation method, the PLGA copolymers with a 50%:50% ratios are particularly successful. The molecular weight is preferably low, 10KD, to middle, ranging from a to 20 KD to 50 KDa.

1.2.4 *P olylactic-co-glycolic acid (PLGA) – Resomer Products*

The Resomers are produced by Boehringer Ingelheim, Germany, in a variety of molecular weights, monomer ratios and end-group functionality. They are largely explored in the pharmaceutical industry, particularly in the design of active agents releasing systems, as well as in the biomedical field. From an end-group functionality point of view, Resomers are produced as uncapped copolymers, with carboxyl end-group functionality and end-group capped consisting of esterified group [6]. The presence of free carboxyl-end groups in the uncapped copolymers renders them more hydrophilic. Thus, the domains made from this copolymer in aqueous suspensions have a more hydrophilic surface than those made of copolymers in which the carboxyl end groups are esterified. This increase in surface hydrophilicity is due to carboxyl-end groups of the polymer chains which orient them toward the external aqueous medium. These copolymers decrease the interfacial tension, thus improving the suspensions stability. The end-capped copolymers have more hydrophobic properties which reduce the stability of aqueous suspensions. The potential of PLGA copolymers for tissue regeneration is great, due to their good biocompatibility and controllable biodegradability. Incorporated in a blend with other polymeric components, this copolymer offers a wide range of possibilities in the preparation of new biomaterials and medical devices.

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1.3 POLY VINYL ALCOHOL

OH   OH   OH
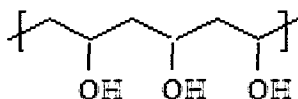
poly(vinyl alcohol)

Poly(vinyl alcohol) is produced through the polymerization of vinyl acetate to poly-vinyl acetate, PVAc, followed by PVAc hydrolysis to PVA. The degree of hydrolysis depends on the time of hydrolysis which never is fully completed, thus resulting PVA as a copolymer of PVA and PVAc. The properties of PVA depend on the degree of polymerisation, degree of hydrolysis and distribution of the hydrolyzed groups.

Poly(vinyl alcohol), is a water-soluble poly-hydroxy polymer with excellent chemical resistance and interesting physical properties. The solubility of PVA depends on the degree of polymerization and degree of hydrolysis as well as on dissolution temperature. The solubility of PVA is hindered by the presence of hydroxyl groups that participate to the formation of intramolecular intermolecular hydrogen bonds [1]. The high number of hydroxyl groups contained in this molecule provides the polymer with the ability to react with a diversity of functional groups and to be part of various polymer blends. PVA is a non-toxic, highly crystalline polymer which has good forming film and great hydrophilic properties. This crystalline polymer is attractive for various applications as a sizing agent in textile, emulsifier, adhesive for paper, and medical applications. PVA offers flexibility, transparency, and toughness [2]. This well known synthetic polymer is

biocompatible and arouses increasing interest in the design of biomedical devices. PVA is amphiphilic and in pharmaceutics it serves as a stabilizer in preparation of microparticles for the delivery of active agents. PVA aqueous solutions are prepared by dissolving polymer powder in water at 90°C followed by cooling at room temperature. PVA gels prepared by freezing and thawing techniques found many pharmaceutical and medical applications. PVA hydrogels have certain advantages thatmake them excellent candidates for biomaterials. They exhibit a high degree of swelling in water and elastic behaviour. PVA gels are promising for their use as soft contact lens material and lining for artificial hearts. It has been shown that PVA can stabilize or destabilize a collidal suspension. Flocculation induced by PVA follows a bridging flocculation mechanism. The PVA has a high affinity for poly-lactic-co-glycolic acid (PLGA), making it the most commonly used stabilizer in formulation of PLGA nanoparticles. As a participant in PLGA-PVA interactions, PVA could play the role of stabiliser, forming a thin layer on the surface of PLGA nanoparticles. In fact, the hydrophobic parts of PVA could become physically entrapped by PLGA polymer chains, thus PVA reacts as a surfactant for PLGA [3]. The hydrogen bonding between PVA and PLGA increases the stability of the structure. These H bonds, known as interpolymer complexes, serve as secondary, physical cross links and provide the networks with additional resistance to PVA dissolution from the structure. PVA is not biodegradable in physiological conditions.

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1.4 CARBON NANOTUBES AS MACROSCOPIC SHAPE BIOMATERIAL AND WET SPINNING PROCESS

The blend of carbon nanotubes with polymers in fibers is advantageous for the creation of new biomaterials containing CNTs. Continuous yarns containing CNT have been produced by electrospining process [1]. This method uses a high-voltage electric field to draw fibers from a polymeric solution through a small nozzle and allows the alignment of SWNTs bundles in the nanofiber containing polymer and carbon nanotubes. Nanocomposite fibers containing silk and carbon nanotubes were produced by the same method with the improvement of mechanical characteristics [2]. In spite of the simplicity of this technique, it lacks in variability of parameters and is deficitary for the production of continuous macroscopic fibers.

Wet spinning is a classical technique which consists in the extrusion of polymer solution into a coagulating bath containing a low molecular substance unable to dissolve the polymer [2]. The contact of polymer solution with the coagulating bath accomplishes polymer precipitation and formation of a coagulated filament. Particle Coagulation Spinning is a promising technique to produce fibers with high carbon nanotube loading [3]. This method consists in the injection of aqueous dispersion of CNTs with SDS in a rotating bath of a coagulating polymer solution such as polyvinyl alcohol. The nanotubes collapse during coagulation and form ribbon-like fibers. The presence of highly exfoliated thin bundles of nanotubes ensures a maximum transfer of their intrinsic characteristics and determines optimal behaviour for the fibers. Several parameters

control the yield of the spinning process and they are related to the spinning conditions, the characteristics of the carbon nanotubes dispersion as well as to the properties of coagulating polymer and its solution [2]. New adapted methods involving the modification of carbon nanotubes dispersion or coagulating bath have been reported, thus enlarging the variety of wet spun CNT-based fibers [3] with various polymer components. Recent works [4] reported the improvement of mechanical behaviour of fibers and their electrochemical characteristics [5]. Using a similar principle, the conversion of CNTs dispersion into fibers has been achieved in a polymer free coagulation bath, by altering the nature of the coagulation bath via pH control. This new approach employs the biomolecules such as hyaluronic acid (HA), chitosan (CH) and DNA as a surfactant [4]. The coagulation bath contains an aqueous solution of diluted acid or divalent cations, such calcium. The spun fibers produced through this approach are robust and flexible, have various shapes and morphologies, and prove to have an excellent electrical conductivity and capacitive behaviour. Their mechanical resistance varies from 90 MPa to 120 MPa while the maximum value of elastic modulus is of 17GPa. These fibers provide a biocompatible support for fibroblast cells, including L-929 cells. Incorporation of carbon nanotubes into the biomedical polymer, such as poly(styrene-β isobutylene-β-styrene), SIBS, shaped in a film demonstrated to posses the dispersive ability of SIBS for SWNTs with improvement of electrical conductivity [6]. When in contact with L-929 fibroblast cells, these films provided a good substrate for cell viability, cell migration and cell orientation due to their intrinsic conductivity.

Recently, carbon nanotube sheets in form of aerogel and yarns, both drawn from MWNTs forest synthesized by catalytical vapour deposition, were tested in contact with a large variety of cells. The results demonstrated their capacity to support long term growth. The highly supportive nature of CNT sheets for directed cellular growth and migration has been demonstrated [7]. Very recently, the biocompatibility of carbon nanotube fibers fabricated from single wall nanotubes by PCS has been investigated in relation to L-929 cells [8]. These fibers, containing carbon nanotubes and polyvinyl alcohol, elicited no significant, acute, cytotoxic effect aftershort-terme exposure. When in contact with PC12 cells, for one week, the fibers demonstrated a good capacity to sustain the growth of cells and to stimulate the formation of neurites.

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1.5. NEURONAL CELLS AND NERVE REGENERATION

1.5.1. Nervous System and Cellular Components

The nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS, which includes the brain, the spinal cord, the optic, olfactory and auditory systems, conducts and interprets signals and provides excitatory stimuli to the PNS. The PNS is composed of all nerve cells and specialized sensory receptors that lie outside the CNS. The peripheral nerves innervate muscle tissues, transmitting sensory and excitatory inputs from and to the spinal column. Most of the information processing, decisionmaking and coordination activities is under the control of the CNS. Two types of cells compose the nervous system: neurons, or nerve cells and neuroglia, or glial cells.

Neurons are the basic structural and functional elements of the nervous system. They are the messenger cells of the nervous systems and communicate with each other through a neuron network. The human body contains more than 10^{11} neurons, each of which makes connections with thousands of other neurons [1]. A typical neuron consists of a cell body with two types of extensions: the dendrites, which branch out in a tree-like fashion and the axon, which is a single fiber that extends over a great distance and branches at the end, giving rise to specialised ending called synaptic terminals. The site of contact between a synaptic terminal and a target cell is called a synapse, the junction

where one neuron communicates with another. The dendrites transmit electrical signals to the neuron cell body while the axon conducts impulses away from the cell body

Glial cells support neurons by carrying out important chemical and physical reactions and by producing a variety of substances that are needed for normal neurological functions. Schwann cells are found in the PNS while astrocytes, microglia and oligodendrocytes are found in the CNS. Schwann cells and the oligodendrocytes form myelin sheaths around the axons speeding the conduction of electrical impulses. Schwann cells play a crucial role in nerve cell regeneration supporting the PNS cells. Oligodendrocytes form protective segments of myelin sheaths around the axons. They extend processes on several axons simultaneously. Astrocytes provide structural support for the neurons, and supply certain substances that are essential for the proper functioning of the nervous system. In the adult brain, astrocytes affect neurogenesis by instructing unspecialized cells to develop into neurons.

Neurons and Signalling

All living cells have an electrical charge difference across their plasma membrane. As a result, the inside of the cell membrane is negative in relation to the outside. The voltage measured across the plasma membrane is called the membrane potential which values range from -50 to -100 mV. Neurons, like any other cell, maintain an electric potential difference across their external membrane, resulting from a differential distribution of electric charges across the membrane at rest, with variations between -40 to -80 mV. Generally, the resting membrane potential in a nerve cell is about -65 mV. Neurons and muscles cells, unlike other cells are excitable; they can alter their resting membrane

potential, measuring between -50 to -55 mV, thus generating a signalling mechanism. The threshold potential in a neuron is typically about 15 to 20 mV. The action potential is the nerve impulse. When a neuron generates a nerve impulse, an electrical message is sent along the axon toward the synapses, which then promote the release of neurotransmiters.

1.5.2. Nerve Growth and Regeneration

The neuronal signalling pathway is directly related to the morphogenesis and differentiation of the nervous system, processes which include the extension of neurites. The survival and development of neuronal cells heavily depends on the action of growth factors. Among them, the Neural Growth Factor (NGF) has the greatest effect. As a response to the signalling from growth factors and as a consequence of the interactions with the extracellular matrix (ECM), the neurites extend out from the cell body. Without the proper activity of the growth factors and an appropriate ECM support, the cells develop in a disorganized manner. These disorganized cells form a glial scar which forms a physical barrier that the regenerating axon can not pierce. Additionally, the glial scar, by both oligodendrocytes and astrocytes, secretes the inhibitory molecules. In fact, recent studies have shown that oligodendrocytes actively block regeneration. A key difference between the nerves of the PNS and the CNS is the capacity of the peripheral nerves to regenerate while the neurons of the CNS do not regenerate naturally. With certain limitations, the regeneration of nerves of the PNS is possible while the CNS will

not regenerate after nerve injury. However, when placed in a proper environment containing growth factors, and in the absence of inhibitory molecules, the neurons regrow [2]. This does not necessarily mean that they regenerate because the barrier is still formed by the glial scar. In this context, the regeneration of nerves of the CNS is still a great challenge. In the case of spinal cord injuries, the complexity is even greater. It seems that there are promising avenues for neural regeneration after spinal cord injury from a clinical point of view. However, there are high limitations for a fully functional recovery. One of the used approaches for the functional nerve regeneration is the guide therapy based on the construction of devices for the guidance of nerve growth. Complementarily to the new development of nanotechnology and the emergence of nanomaterials, tissue engineering is able to apply new approaches to promote an enhanced functional regeneration.

1.5.3. Neural Biomaterials

Neuro-materials, or neural materials, are typically soft polymers or their composites. They possess specific characteristics which should be considered when designing new neural biomaterials as guides for the stimulation of the regenerative capacity of injured nerves. Among them, biodegradability, flexibility and surface texture are the main requirements. Moreover, a reduced swelling capacity of such a material is suitable [3]. Polymers are largely used for the design of neural guides and neural prostheses. Silicone was one of the first materials to be used for the nerve guide[4]. However, the lack of

biodegradation and the potential to swell in water are serious limitations. Other polymers such as poly-pyrole were retained for their electrochemical properties which stimulate the proliferation and differentiation of neural cells. In combination with biopolymers, this material provides a good potential for the regeneration of neurons. Biodegradable polymers such as polylactic-co-glycolic acid and poly-caprolactone proved great potential for their ability to stimulate the regenerative capacity of nerve cells [5] .

New potential of innovative therapies has recently been identified for neural regeneration. The emergence of carbon nanotube structures and their potential for nerve regeneration is one of these new alternatives. An exploration of these new structures along with their potential for biotechnology is of enormous interest and constitutes the topic of several research groups.

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CHAPTER 2

OBJECTIVES AND RELATION WITH THE PAPERS

The research work of this thesis focuses on the development of new biomaterial containing carbon nanotubes with biocompatible and biodegradable behaviour when in contact with living cells. In chapter two, the state-of-the-art of CNT materials in the area of bio-nanotechnology reveals the enormous potential of carbon nanotube (CNT) structures for medical and biological applications and the interest in this field. The growing interest for the efficiency of CNT as a neural biomaterial is presented in the context of the very recent studies that demonstrate the capacity of CNTs to help nerve growth.

The general goal of this project is to design CNT-based fibers as neural biomaterials, to preferentially guide the growth of neural cells and to stimulate their functions. In this perspective we have identified that the wet spinning process is ideal, as it allows the integration of nanotube structures in a macroscopic shape. To address this issue four specific objectives were formulated:

    i)   Design an experimental model based on the Nanotube-Sphere Binary Colloidal Systems (NSBCS) used as spinable mixtures;

    ii)   Formulate and fabricate CNT macroscopic fibers by the wet spinning process;

    iii)   Identify and evaluate the specific characteristics of these new fibers ;

iv)   Investigate the potential of these fibers as new biomaterial and prove their biocompatibility and their biodegradability in relation with neural cells and fibroblasts.

Macroscopic fibers containing CNTs have been successfully fabricated by the Particle Coagulation Spinning method which spun fibers from CNT into aqueous dispersion. Our strategy therefore is to maximally exploit the potential of this wet spinning process in order to develop a new method for the fabrication of macroscopic fibers by a hybrid approach. The success of this project lies in the spinning of a binary colloidal mixture endowed with an improved spinnability and high potential for the modulation of fiber characteristics. The hybrid approach consists in mixing CNT with an organic component such as copolymer. In the same light the rod-like geometry of carbon nanotubes is combined with the spherical shape of polymeric nanoparticles in order to create new spatial structures and to induce the biodegradability.

In the first step the new dispersion is designed as a colloidal mixture and a new component is identified, in the polylactic-co-glycolic-acid (PLGA) which precipitates as spherical nanoparticles suspended in water. This choice considers the biocompatibility and biodegradability as two key goals to be reached. The PLGA copolymer is easily disolved in non toxic organic solvents, partially miscible with water, thus avoiding the phase separation behaviour. The formation of copolymer nanoparticles by nanoprecipitation is a well-known technique in the pharmaceutical process and allows the precipitation of copolymer from organic solution in water, thus forming an aqueous suspension with the pseudolatex characteristics.

The second step consists of the preparation of the binary colloidal mixture by mixing a CNT aqueous dispersion with a PLGA aqueous suspension. This step is decisive for the design of the fiber structure, as the copolymer characteristics determine the mixing process. An experimental model has been established for single wall nanotubes and multi wall nanotubes respectively, by taking into consideration the volume ratio of each component in order to obtain a stable colloidal mixture with high spinnability.

The development of the spinning process is the third step of the work and includes the injection of a colloidal mixture into the coagulating bath followed by the coagulation of the mixture. The characteristics of the latter highly influence the formation of a monofilament thread in the coagulation bath. Through this step we accomplish the second specific objective.

The achievement of the third specific objective entails the general characterisation of fibers and asseses their biodegradability. In this fourth step six distinctive characteristics are identified in relation to the biocompatibility response: internal structure, surface characteristics, bulk composition, mechanical properties and visco-elastic behaviour. The biodegradability aspect is mainly controlled by the amount of copolymer contribution and is related to fiber composition.

The proof of biocompatibility is the fifth step of this work and focuses on the fourth specific objective. This step consists of the general assay in vitro, for testing fiber response in contact with neural cells and fibroblasts. The first type of cells is directly

related to the biofunctionality we design while the second type is related to the contact of these fibers with the extra cellular matrix.

The design and development of this research project were possible through an extended investigation of the carbon nanotube structure and the challenges related to their fabrication, particularly for medical applications.

Chapter1 emphasizes the use of CNT structures in the area of bio-nanotechnology and the aspects related to the biocompatibility. The polylactic co-glycolic acid is presented as a biomaterial focusing on the interest to prepare PLGA nanoparticles through a nanoprecipitation method. The end of this chapter is dedicated to the presentation of neural cells and the main aspects of nerve regeneration. Chapter 2 focuses on the objectives of the project and the work strategy developed in the main five steps. The primary aspects of the experimental protocol and the techniques used in the experimental work are described in Chapter3. Chapter4 is concerned with the innovative contributions of this work consisting of the elaboration of new fibers by spinning from a binary colloidal mixture. This paper presents the proof-of-concept results to prove the biocompatible behaviour. Chapter 5 centers on the aspects related to the integration of nanotubes into macroscopic fibers and illustrate the main effects of this integration: the hybrid integration of nanotubes, the effect induced at nanoscale and consequences on the contact with cells. The description of fiber characteristics is is part of Chapter 6 and demonstrates the efficiency of the new formulation while revealing the role of the surface in the interaction of fibers with cells. The general discussion presented in Chapter 7 underlines the original contributions of this research and their multiples

advantages in the design of dynamic substrates for the interactions with living cells. Finally we conclude on the main achievements of this work and present some perspectives.

CHAPTER 3

EXPERIMENTAL TECHNIQUES

3.1 DISPERSION OF CARBON NANOTUBES

Dispersion of Single-Wall Carbon Nanotubes in Sodium Dodecyl Sulphate: D-SW

Single-wall carbon nanotube purified powder (lot PO 257, PO 272), synthesized by Gas-Phase Decomposition of CO (HiPCO), was purchased from Carbon Nanotechnologies Inc.

To single-wall carbon nanotubes (0.03g, 0.3 wt.%) was added sodium dodecyl sulphate (SDS) (0.10g, 1.0 wt.%) and water (9,87 g, 98.7 wt.%). The aqueous mixture was then ultrasonicated using a sonicator horn over a period of 60-80 min at 40KW

Dispersion of Multi-Wall Carbon Nanotubes in Sodium Dodecyl Sulphate: D-MW.

Multi-wall carbon nanotubes (lot NTC 3056), synthesized by catalyzed Chemical Vapor Deposition (CVD), were purchased from Arkema. To multi-wall carbon nanotubes (0.18g, 0.9 wt.%) was added sodium dodecyl sulphate (0.24g, 1.2 wt.%) and water (19,58mL, 97.9 wt.%). The aqueous mixture was then ultrasonicated, using a sonicator horn over a period of 45-60 min at 20KW to obtain a homogenous dispersion

3.2 PREPARATION OF AQUEOUS SUSPENSION OF PLGA

PLGA identified as Resomer RG 503H (RG503H) and Resomer RG 502 (RG 502), comprising a 50:50 ratio of lactic: glycolic acid, were purchased from Boehringer Ingelheim (Ingelheim, Germany). RG 503H has a viscosity of 0.32-0.44 dlg^{-1}, and middle molecular weight, MW of 34 KDa. RG 502 has a viscosity of 0.16-0.24 dlg^{-1}, and a low molecular weight, MW of 12 KDa. Both copolymers were solved in acetone to obtain PLGA solution. Two solutions were prepared for each Resomer: 1) 200 mg were dissolved in 13 mL acetone; 2) 600 mg were dissolved in 13 mL acetone. 12 mL distilled water were added dropwise to each solution for nanoprecipitation. The blends were stirred at room temperature for complete evaporation of acetone, to yield 12,5 mL aqueous suspensions: S1-RG503H, S2-RG503H and S1-RG502, S2-RG502, respectively.

3.3. PREPARATION OF COLLOIDAL MIXTURE

<u>SWNT Colloidal Mixture</u>

2 mL of S1-RG502 and S2-RG502 were added to 1 mL SW dispersion (D-SW) to obtain 3 ml of M1-SW- RG502 and M2-SW-RG502 mixture. 3 mL of S1-RG503H, S2-RG503H were added to 1 mL SW dispersion to obtain 3 ml of M1-SW-RG503H and M2-SW-RG503H mixture.

MWNT Colloidal Mixture

7 mL of S1 and S2-RG502 were added to 1 mL D-MW to obtain 8 ml of M1-MW-RG502 and M2-MW-RG502 mixture. 5 mL of RG503H-1, RG503H-2 were added to 1 mL MW dispersion to obtain 6 ml of M1-MW-RG503H and M2-MW-RG503H mixtures. The mixtures were sonicated 15 min for homogenization. Each mixture was sonicated over a period of 15 min to obtain a homogenuous mixture.

Dispersion-like Films

Dispersion-like films were prepared from each dispersion of SWNTs and MWNTs and their various mixtures in order to identify the effects related to the presence of nanoparticles.

The films were formed by the casting of a mixture from a manually operating syringe. The film is spread onto the glass slide and stays in contact with air for water evaporation. Through this operation, we tray to attain a state of dispersion close to the proto-fiber status and are thus able to evaluate the characteristics similar to that of the mixture when it exits from the needle. A clean glass slide was used as a substrate for manual cast-coating. An inclination of the glass slide at an angle of 45° avoids spreading and converges the dispersion into a stream, similar to the injection in the coagulation bath. After water evaporation, these dispersion-like films include arrangements of nanoparticles with nanotubes, similar to the internal structure of the corresponding fiber, as viewed in cross section imaging. The texture of each film was examined by SEM and new domains were identified as a function of the suspension.

3.4. PREPARATION OF 5% PVA AQUEOUS SOLUTION

Polyvinyl alcohol powder was purchased from Sigma-Aldrich (Lot 70580); polyvinyl alcohol having a hydrolysis percentage of 88-89% and MW of 150 KDa. Distilled water (950 mL) was added to PVA (50g) and the mixture was heated to 95°C while stirring. After 45 min, the mixture was cooled to room temperature, while stirring to yield a 5% PVA aqueous solution.

3.5. SPINNING PROCESS

Injection of the Colloidal Mixture into PVA

A PVA solution (150-200 mL) was placed in a coagulation bath (glass cylinder of 80-100 mm in diameter and 70-90 mm in height and fixed concentrically on a rotating table). A stainless steel needle (0.50 mm I.D) was used for injecting the dispersion into the coagulation bath. This needle was bent so that the dispersion could be injected parallelly to the bath surface. The point of injection of the dispersion was at a radius of 20-30 mm from the center of the cylindrical dish, about 10-20 mm under the surface of the PVA solution, and parallel to the dish bottom. The glass cylinder containing PVA solution was rotated at 40-60 rpm. The rate of 0.80-0.85 mL/min for injecting the dispersion into the coagulation bath was achieved using a syringe pump. After laminar rotational flow was established, the syringe pump was activated and the dispersion (5 mL) was injected in a direction parallel to the established flow. The coagulation of the

dispersion formed a continuous spiral ribbon inside the PVA solution. The ribbon was subsequently washed in water and air dried to form carbon nanotube-based fibers.

Rinsing and Drying of Carbon Nanotube-based Fibers

The carbon nanotube-based fiber was carefully transferred from the PVA solution into a bath of distilled water and left there over a period of 30 minutes with no stirring or agitation. This procedure was repeated three to six times, each time using a fresh bath of water. The washed ribbon was then suspended and dried under ambient conditions.

3.6. CHARACTERISATION OF CARBON NANOTUBE-BASED FIBERS

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy was performed using a Hitachi System, Model S-3550N. The topography and surface morphology of each carbon nanotube-based fiber was observed. More specifically, the carbon nanotubes alignment and arrangement with copolymer nanoparticles was investigated.

Atomic Force Microscopy (AFM)

Atomic Force Microscopy was performed using a Nanoscope III A controller, a multimode AFM head (Digital Instrument Santa Barbara CA, USA) using a silicon cantilever. The imaging was performed in taping mode on a fiber surface for a selected area of 500x500nm^2, 5x5 μm^2 and 10x10 μm^2, respectively. The AFM amplitude,

topography, and phase scans were investigated for the selected areas of each carbon nanotube-based fiber.

Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis was performed using a Setaram instrument, a high-performance Modular Thermogravimetric Analyzer, TGA, at a rate of 10°C/min under argon atmosphere at a maximum temperature of 600°C. The composition of each carbon nanotube-based fiber was determined.

Dynamic Mechanical Analysis (DMA)

Dynamic Mechanical Analysis was performed using a DMA Perkin-Elmer 7E Analyser, in tensile mode under isochronal conditions at a frequency of 1 Hz. A force of 20 mN was applied to the carbon nanotube-based fiber while the temperature scans were ramped from 20°C to 110°C at a rate of 5°C/min. The modulus (stiffness) and damping (energy dissipation) properties of the carbon nanotube-based fibers were investigated under oscillatory stress. The loss modulus (G''), storage modulus (G') and loss factor (Tan δ), as well as the ratio of G'' and G', were determined for each carbon nanotube-based fiber

Mechanical Behavior - Tensile Test

The mechanical behavior of CNT-based fibers was investigated using an Instron 4301 testing machine in tensile mode. The stress (σ), strain (ε) and Young's modulus (E)

were determined for each carbon nanotube-based fiber (Data illustrated in Table 4. 2).

Biodegradation of the Carbon Nanotube-based Fibers

The biodegradability was assessed over a 6 week period in phosphor buffer saline (PBS). Nanoscale morphology and topology were investigated using Low-Vacuum Scanning Electron Microscopy (LV-SEM) equipped with an energy dispersive detector (EDS).

3.7. *in vitro* BIOCOMPATIBILITY

The capability of the carbon nanotube-based fibers to influence the growth and proliferation of living cells was tested in culture with PC12 cells and Human Skin Fibroblasts.

i) PC12 cells

PC12 cells were used to investigate the ability of carbon nanotube-based fibers to stimulate the proliferation of nerve cells, to modulate their growth process and to contribute to the extension of neurites. These experiments demonstrate the potential use of the carbon nanotube-based fibers as neural biomaterials for the regeneration of neural cells, particularly to facilitate nerve regeneration for injured parts of the spinal cord.

Culture

PC12 cells, rat pheochromocytoma cells, are usually employed as a model system for neuronal development studies. Cultured in a medium containing animal blood serum, the PC12 cells adopt a round and phase bright morphology and proliferate to high density. Cultured on collagen coated plates, the adhesion to the substrate is enhanced. These

cells can cease proliferating and undergo differentiation in the presence of specific trophic substances, such as neural growth factors (NGFs). The formation of neurites serves as indicator for cell differentiation. Four aspects were studied: adhesion, migration, proliferation and differentiation of the PC12 cells.

Substrate Samples

The carbon nanotube-based fibers containing SWNTs and MWNTs were used in the bio-assays. Pre-cleaned microscope glass slides (Erie Scientific, lot No. 2951) of 1 mm thickness were used. Rat PC12 cells, obtained from American Type Tissue Collection, were used in the cell cultures. Since rat PC12 cells are known to have poor adhesion to plastic, a collagen coating was used to improve adhesion. Type II collagen, purchased from Sigma-Aldrich Canada Ltd., was used to coat the plates and/or as solution for fiber immersion. RPMI Medium 1640, containing 10% heat-inactivated horse serum and 5% fetal bovine serum, was purchased from Gibco –BRL (Grand Island, NY, USA), and used as growth medium. Phosphate Buffered Saline (PBS) was purchased from Sigma-Aldrich Canada Ltd. The culture medium was supplemented with 50ng/mL Neural Growth factors (NGF- Gibco –BRL, Grand Island, NY, USA) to induce cell differentiation.

Procedure for Sample Preparation

Microscope slides were cut into pieces (7mm x 7mm), using a diamond cutter. The cut slides were then cleaned in an ultrasonic bath, 20 minutes in acetone, followed by 20 minutes in distilled water. The fiber sample, comprising 20 carbon nanotube-based

fibers, was supported on the 7x7 mm^2 glass slide. The control sample was a collagen coated 7x7 mm^2 glass slide. The samples were placed in a 12-well tissue culture plate, which was sterilized with ethylene oxide (EO) gas at 37 °C following standard procedures, and washed once with phosphate buffered saline (PBS). For adhesion studies, the samples were placed in a 12-well tissue culture plate coated with a 0.5 mg/mL solution of sterilized Type II Collagen, and sterilized for 45 minutes.

Procedure for MTT Assay

The MTT test is a colorimetric metabolic assay enabling a quantification of cellular growth through changes in cell proliferation, cell viability and cytotoxicity as a response to external factors. The assay is based on the capacity of various living cell dehydrogenases to cleave MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and display a dark blue formazan product. The formazan product is mostly impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The process requires active mitochondria to cleave significant amounts of MTT, the yellow tetrazolium salt (MTT) is reduced to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The number of surviving cells is directly proportional to the level of the formazan produced, allowing spectrophotometric procedures to detect changes in cell metabolism. The results were read on a multiwell scanning spectrophotometer (ELISA reader), and the relationship between cell number and absorbance was established.

After a three day culture period, the cells were transferred from the individual wells to 12 mL Sarted tubes, with 2 mL culture medium. The culture medium was centrifuged for 10 minutes and the supernatant was removed. The cells were then subjected to the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. A 5 mg/mL MTT stock solution in phosphate buffered saline (PBS) was prepared. To each culture tube 0.2 mL of MTT stock solution was added followed by incubation at 37°C. After 4 h, the supernatant was removed and 0.2 mL of color development solution (0.04N HCl/isopropanol) was added to each well before continuing the incubation for an additional 15 min. The HCl converted the phenol red to yellow, which does not interfere with MTT formazan measurements. Formazan was dissolved by isopropanol and produced a homogeneous blue solution suitable for absorbance measurements. When the cells broke, the product (formazan) turns purple, turning the solution purple as well. Three samples of 200 µL of the purple solution were transferred from each well to a 96 flat-bottom well plate. The optical density was measured with the ELISA plate reader (model 680, BioRad Laboratories, Mississauga, ON, Canada) at 570 nm. The absorbance was proportional to the number of living cells present. The value of absorbance (Optical Density, OD) was measured for each type of fiber and it represents the viability of the cells.

Observation of Cultured PC12 Cells

Cell Observation with Inverted Microscope

A Nikon Eclipse E800 microscope, model Nikon, Corp., Yokohama, Japan, equipped with a digital camera for image registration was used to observe the carbon nanotube-based fibers. The carbon nanotube-based fibers were not optically transparent, preventing the visualization of cells attached to the fiber by optical microscopy. However, the images taken with the inverted microscope, at the fiber/cell interface, displayed the orientation and the presence of cells on the fibers.

Cell Observation with Scanning Electron Microscope (SEM)

The microscopic observation was performed using a Variable Pressure Scanning Electron Microscope, model S-3500N, Hitachi. After three days of culture, the samples were washed three times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min. The fixed cells were washed with distilled water and gradually dehydrated in ethanol.

Cell Visualization by Hoechst Staining

Hoechst staining was used to identify the cells attached to the membrane as well as to identify the formation of neurites. After three days of culture, the samples were washed three times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min. The samples were then incubated at room temperature for 15 min in 2 mL of 2µg/mL Hoechst solution (Hoechst 33342/PBS). The observation of stained cells was performed with an epifluorescence microscope (model Axiophot, Zeiss, Oberkochen, Germany). A qualitative analysis of

the florescence images demonstrated cell growth, neurite formation and confirmed the contribution of the fibrous substrate to cell adhesion.

Procedure for PC12 Cell Adhesion and Growth

Cell adhesion was tested for the carbon nanotube-based fibers of the present specification using the previously prepared samples. For one series of samples, adhesive capacity was increased, using collagen coated plates, to avoid agglomeration of cells. The PC12 cells were cultured in the presence/absence of neural growth factor (NGF). The cells were imaged using florescence microscopy to demonstrate the presence of cells on the fibers and their capacity to extend neurites in the presence of neural growth factors (NGFs).

The capacity of the carbon nanotube-based fibers to encourage the migration of cells was evaluated by locally seeding PC12 cells, in 0.05mL of culture media, on the sample. Visualization of the samples by light microscopy demonstrated the migration of cells as function of the type of fiber; migration of PC12 cells from the initial position, where the cells has been seeded, to the opposite side of the sample is visualized by microscopy.

ii) Human Skin Fibroblasts

Human skin fibroblasts were used to investigate the ability of carbon nanotube-based fibers of the present specification to form a fibrillar network of the extracellular matrix to sustain cell proliferation. These experiments demonstrate the potential use of the carbon nanotube-based fibers of the present specification as biomaterials for the attachment, alignment and

proliferation of cells.

Human Skin Fibroblast Culture

Fibroblasts are generally known as anchorage-dependant cells. Pre-cleaned microscope glass slide (Erie Scientific, lot No. 2951) of 1mm thickness were used. Human skin fibroblasts were obtained from Clonetics (San Diego, CA, USA), and used in the cell cultures. Fibroblast growth medium was composed of Dulbecco's Modified Eagle (DME) medium supplemented with 10% Fetal Bovine Serum (FBS) purchased from Gibco (Burlington, Ontario, Canada) and penicillin G purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Phosphate Buffered Saline (PBS) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Procedure for Sample Preparation

The procedure for sample preparation was similar to the one described for PC12 cells. The microscope slides were cut into pieces (7mm x 7mm) using a diamond cutter. The cut slides were then cleaned in an ultrasonic bath, 20 minutes in acetone, followed by 20 minutes in distilled water. The fiber sample, comprising 20 carbon nanotube-based fibers, was placed on the 7x7 mm^2 glass slide. The control sample was a 7x7 mm^2 glass slide coated with a RG502-1 film and a PVA film. The samples were placed in a 12-well tissue culture plate, which was sterilized with ethylene oxide (EO) gas at 37°C following standard procedures, and washed once with phosphate buffered saline (PBS).

Human skin fibroblasts were seeded in 12-well plates, density of 2.5×10^4 cells /cm^2, and cultured for 3 days at 37°C, 90% humidity and under a CO_2 atmosphere.

Procedure for MTT Assay

The procedure was the same as the one described for PC12 cells. The variation of absorbance value (Optical Density, OD) as a function of the carbon nanotube-based fiber. The MTT values demonstrate the capacity of the single-wall carbon nanotube-based fibers and multi-wall carbon nanotube-based fibers of the present specification to support cell proliferation. It is suggested that the proliferation of the fibroblasts was promoted by fibers comprising a higher percentage of copolymer.

Observation of Cultured Human Skin Fibroblasts

The cultured fibroblasts were observed using the same techniques and procedures as described for PC12 cells (*i.e.* inverted microscopy, scanning electron microscopy and Hoechst staining).

Procedure for Human Skin Fibroblast Adhesion and Growth

Cell adhesion was tested for carbon nanotube-based fibers. Inverted microscopy images of cultured cells demonstrated their adhesion to the fibers and their growth along the fibers,

Florescence microscopy demonstrated uniform cells spreading along fibers after 3 days.

The capacity of the carbon nanotube-based fibers to incite cell migration was evaluated by locally seeding human skin fibroblasts. Visualization of the samples by light microscopy demonstrated the migration of the cells in function of the type of fiber. We observed the migration of human skin fibroblasts from an initial position to an opposite side of the sample.

CHAPTER 4

DESIGN OF FIBERS SPUN FROM CARBON NANOTUBE- SPHERE

BINARY COLLOIDAL SYSTEMS AS SUBSTRATE

FOR CELL'S BEHAVIOUR CONTROL

TITLE PAGE

DESIGN OF FIBERS SPUN FROM CARBON NANOTUBE- SPHERE

BINARY COLLOIDAL SYSTEMS AS SUBSTRATES

FOR CELL'S BEHAVIOUR CONTROL

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4.1 ABSTRACT

The relevance of carbon nanotubes (CNTs) for the future of regenerative medicine is centered on harnessing their multifunctional character to design nano-sized devices and to spawn specific interactions for promoting advanced therapeutic approaches. This work is directed toward building a new biomaterial containing CNTs by using the Particle Coagulation Spinning (PCS), a method allowing the fabrication of structured fibers in view of developing dynamic and safe biomaterials. Capitalizing on the wet spinning process, by a hybrid approach, we integrate CNTs in macroscopic fibers with biodegradable and biocompatible responses. The fibers with variable compositions show an intricate structure and unique morphology that provide the capacity to stimulate and sustain cell proliferation, particularly for neural cells, offering an efficient substrate for cell differentiation. These structures are ideally suited to direct cellular growth and for future tissue engineering applications under the form of fibers and weaven or non-woven textiles.

4.2. NANOTUBE-SPHERE BINARY COLLOIDAL SYSTEMS (NSBCS)

Carbon nanotubes [1] are tubular arrangements of sp^2 hybridized carbon atoms and represent ideal building blocks for a wide range of materials with great potential for biotechnology. The pertinence of these tiny entities for regenerative medicine is centered on their multifunctional character allowing to spawn specific interactions and to create miniaturized devices for advanced therapeutic approaches [2,3]. Studies on these cylinder-shaped macromolecules suggest that CNTs are ideal for modulation of interactions with cells [4,5]. Several experimental works revealed the ability of CNTs to help the growth of neural cells [6,7] and proved their capacity to contribute to the restoration of damaged neuronal circuits [7,8,9,10,11,]. Moreover, it was confirmed that the excellent electroactivity of nanotubes stimulates the growth of cells and greatly improves the neuronal performance [12,13,14,15]. However, these works did not address the issue of biodegradability. In addition, the processing of CNT-based materials is a central issue since CNTs have to be structured under optimal macroscopic forms and combined with various bio-active components. CNTs' potential for cell regeneration lies in their electrical conductivity, chemical inertness and their physical characteristics: they are light-weighted, have a large specific area and an outstanding aspect ratio and they mimic the collagen structure [16,17,18]. However, some previous works have proved that the handling of the CNT's pristine is associated with potential toxicological effects [19,20]. We face new biocompatibility perspectives while a great challenge is associated

with the concatenation of nanotubes in macroscopic structures, without loosing the outstanding properties conferred by the nanoscopic dimensions [21]. Since, the size and shape matter seeded a big controversy regarding the quest of CNTs [22]. Their integration into nanocomposites [23] has become a great challenge for the development of new safe biomaterials. Recently, research endeavours focused on new methods for the processing of CNTs [24,25,26]. The wet techniques appear to be preferable for the purpose of a biomaterial.

Here we report the creation of CNT-based fibers using a hybrid approach, while maximizing the efficiency of the wet spinning to integrate CNTs in macroscopic hybrid fibers. Our material is a harmonized combination of dispersed CNTs, a polylactic-co-glycolic acid (PLGA) copolymer and polyvinyl alcohol (PVA). The incorporation of PLGA nanoparticles underpins the fiber structure, allowing the arrangement of nanotubes in the nanoparticles' lattice. A synergistic contribution of PLGA and PVA greatly improves the fabrication process and enhances the functionality of CNTs.

Shaping CNT-biomaterials through a wet spinning process in aqueous media is very advantageous for the engineering of biomaterials [28,29,30]. Here, we present the fabrication of a new biomaterial containing CNTs and shaped as a fibrous complex structure using PCS [31], a method which eludes the CNT's covalent chemistry. Our concept is based on the development of a spinnable Nanotube-Sphere Binary Colloidal System (NSBCS) in a wet spinning process. It contains CNTs dispersed with sodium

dodecyl sulphate (SDS) and an aqueous suspension of PLGA nanoparticles combined in a variety of ratios. The efficiency of this method resides in the synergistic effect of spherical nanoparticles and rod-like particles assembled in a binary colloid system which plays a major role in the spinning process. The macroscopic fibers we produce have variable contents of CNTs, Table. 4.1, merging with PLGA nanoparticles, both intertwined with PVA to form fibrilar units, Fig. 4.1-2a.

4.3. WET SPINNING

We are working in aqueous media without using chemical reagents in order to provoke or intermediate some chemical changes. Hence, the modifications that take place are exclusively provoked by the native state of each component and the interactions between them. Moreover, the presence of an aqueous coagulant medium results in the material's cleanness and allows the introduction of numerous active agents or biological molecules without their denaturation. This is an essential opportunity for the application of pre- or post-treatments in order to manufacture complex hybrid biomaterials containing CNTs. The single wall carbon nanotubes (SWNTs) or multi wall carbon nanotubes (MWNTs) processed by this method result in a monofilament thread of variable diameter, Fig.4.1-3a, 3d., and length. We identify a homogenous phase distribution on the surface, along the fiber, Fig.4.2-1, and across the fiber, Fig. 4.2-2.

For CNTs, the dispersion state and nanotubes' distribution in the macroscopic shape are critical factors influencing the cellular exposure to CNTs [16,17]. CNT dispersion involves wetting out and disrupting the agglomerate of nanotubes, thus producing a suspension of particles with an optimal size distribution [32,33]. We decided on the aqueous process as ideal for the fabrication of CNT-based biomaterials. Previous studies have shown the possibility to spin fibers from aqueous suspensions of SWNTs or MWNTs [31,34]. Nanotubes are first dispersed in water, stabilised by surfactants and then injected into a coflowing stream of a coagulating polymer solution.

The introduction of PLGA suspension is the key stone of this work. Prior to the mixture, the copolymer is shaped in nanospheres, Fig. 4.4-2, by a nanoprecipitation procedure [35]. The choice of copolymer is supported by its biodegradability and its FDA approved biocompatibility. The molecular weight of the copolymer and its end-chain architecture are variables of the process. The end-chain architecture describes the reactivity of the copolymer while the molecular weight plays a crucial role in the control of the biodegradability rate. We use two random copolymers each with different molecular weight, such as Resomer RG502 and RG503H, with low and middle molecular weight, respectively. The latter is an end-group uncapped chains copolymer, with free carboxyl groups, while the first has end groups capped chains. Measurements of the glass transition temperature (T_g) of two amorphous copolymers indicate a value of $38°C$ for RG503H, and a value of $41°C$ for RG502. The preparation procedure is free

from surfactant and results in a quasi-stable suspension. Measurements of the mean particle diameter, using Dynamic Light Scattering (DLS) method, show the particle's size and prove the monodispersity of the suspensions, Table 4.2. These dimensions were also confirmed by AFM measurements. Suspended in water, these sphere-like particles mixed with dispersed CNTs form homogenous dispersions, Fig. 4.3-1. We combine CNT original dispersion, Fig. 4.4.-1, with PLGA aqueous suspension, Fig. 4.4 -2, with the aim to generate a colloidal mixture, Fig. 4.4-3. The later plays a main role in the design of a diversity of fibers by the choice of different components and variation of their proportions. The ratio of combination of SWNTs, in volume, is of 2:1 for RG502 copolymer, and of 3:1 for RGH503 copolymer. In the case of MWNTs this ratio is of 5:1 for RG502 copolymer and of 7:1 for RGH503 copolymer.

The resulted mixtures are very homogenous and stable and no phase separation behaviour appears. The distribution of nanotubes between spherical particles is described in Fig. 4.4- 4. A close observation of film-like dispersions provides the details of the colloidal mixture before its contact with the coagulating bath. Particular orderings of nanoparticles have been identified, Fig. 4.1-1, with a perceptible role in the formation of fiber structure, as described by the fiber cross sectional micrographs, Fig. 4.1-2. This information suggests a net difference between dispersed SWNTs and MWNTs in their relation to co-polymeric spheres. They all have associative abilities, Fig. 4.1-1, and strongly influence the spinning process, with the effect on the fiber texture, Fig. 4.1-3. The diversity of the spheres in size, composition and concentration provides a great

versatility for the binary system. Reciprocally, in these arrangements, each type of nanotube offers various topological feedbacks, Fig.4.3-1.

The configuration of the mixture dispersions at sub-microscopic and microscopic level is related to the spatial confinement created by the gaps between spherical particles. They ensure the insertion of nanotubes in the PLGA lattice, Fig. 4.4- 4, and assist the formation of the fiber during the spinning process, Fig. 4.1-1. Two types of spatial confinements are imposed through the fiber's process. The first is related to the preparation of mixture and stems from the tendency of nanospheres to organize in lattices with gaps inserting nanotubes, Fig. 4.4-4, thus resulting in a homogenous mixture. The nanotubes' segmental mobility is accompanied by their migration from the sphere walls and finally leads to an enhanced concentration of nanotubes in the center of interstices, Fig. 4.4-4. This association between nanospheres and nanotubes is maintained into the syringe, Fig. 4.5-a (1), and slightly changes during the passage in the syringe's needle. The second restriction is related to the passage of the mixture from the cylindrical syringe's needle, to the conical nozzle, characterized by a 500 µm input diameter and 300µm output diameter, Fig. 4.5-b (2). At this stage of the process, the combination of the confinement effect with the shear flow action is advantageous. Due to the ratio between nozzle's diameters, the mixture is progressively forced into a more ordered structure. Working at room temperature, below the PLGA glass transition temperature, the spheres are not deformed, but they are rather closely packed, thus enhancing the contact with nanotubes. In these conditions, the orientation of spherical

particles goes along with the alignment effect induced by nanotubes which play the role of anisotropic inducers, Fig. 4.5-b (2). Additionally, the end-to-end connections of nanotubes with spherical nanoparticles take place in some situations resulting in the formation of colloidal arrays with a pearl-like morphology, Fig. 4.3-1. These strengthened domains prevent the nanotubes from sliding due to their smooth cylindrical outer surface and endows the mixture with an improved spinnability. This results in a continuous macroscopic fiber with a uniform internal structure, Fig. 4.3-2, and a periodic textured surface, Fig. 4.6-2. In this hybrid formulation, the participation of copolymer nanoparticles limits the bias in the alignment of CNTs, Fig. 4.3-2 (b), and favours the preferential alignment in the direction of the flow, Fig. 4.1-1.

In the wet spinning process, a dope solution is extruded through the small hole of a spinneret immersed in a liquid bath. Mutual diffusion between the freshly formed fluid filament and the coagulating bath causes the solidification of the dispersion by the coagulation process which steers the fiber's structure [37]. This process begins during the first seconds of contact when the surface of the filament coagulates first, followed by the formation of an interface between coagulated and non coagulated layers; this interface moves to the core region during the mutual diffusion process. The design of this new method relies on the original PCS [36]. The method we propose promotes the spinning of CNT macroscopic fibers from a binary colloidal mixture containing CNTs combined with PLGA nanoparticles in a variety of ratio, thus resulting fibers with various CNT content, Table 4.1. The parameters of the spinning process we perform are

similar to that of original PCS [31]. However, by using a binary colloidal system, we generate new conditions for the coagulation mechanism. The colloidal mixture prolongs its performance as a many-particle system at its entry in the coagulation bath, Fig. 4.5-2. While in the original method, the coagulation process goes forward with the first protrusion of CNTs in the PVA solution and bridging coalescence arises, in the new method, the coagulation emerges in two steps: i) the first contact is controlled by the adsorption of PVA on PLGA due to their affinity [37] ; ii) the bridging between CNTs, nanospheres and PVA chains follows and the real coagulation phenomena takes place, Fig. 4.5-2. Consequently, the inner structure of new fibers is very different from that of original fibers and does not show skin-core structure, Fig. 4.1- 2b. During the mixture injection, the proto-fibers are formed and further protrude the PVA stream to coagulate and form a tertiary blend organized in a fibrous monofilament thread with variable texture, Fig. 4.6-2.

The washing stage follows after the thread was pulled out of the bath and its efficiency is strongly influenced by the immiscibility of PLGA with water. The higher the number of washing cycles is, the greater the removal of PVA, thus favouring the presence of nanoparticles on the surface. The drying of this thread by dangle gives rise to a continuous yarn with a cylindrical shape, Fig. 4.1-3a, 3d. PLGA nanoparticles improve the quality of the evaporation process thus yielding a more compact structure. The fiber's internal structure, Fig.41-2, and its external profile Fig. 4.6-2, are directly

related to this new formulation and are marked by the presence of PLGA in its spherical shape, Fig. 4.6-1. Both the size of nanoparticles and the spatial placement of these spherical shapes determine the roughness of the fiber's surface.

4.4. INVESTIGATION OF FIBERS

By initiating the suspension of PLGA nanoparticles, we aim to tailor the characteristics of new fibers, particularly their biodegradability and their biocompatibility. Using the PLGA, we palliate the negative impact of carbonaceous species originating from CNT synthesis, thus providing a favourable effect on the hybrid system. The incorporation of PLGA spherical nanoparticles roots the structuring of proto-fibers, thus improving the spinnability of the mixture for the fabrication of macroscopic threads, Fig. 4.1-3a, 3d.

Thanks to PLGA, the fibers become partially biodegradable, a performance that could be tuned varying the copolymer's ratio and its properties. This is an important achievement, considering that neither CNTs nor PVA are biodegradable in physiological conditions [3]. Inserted between nanospheres and not adsorbed onto their surface, the nanotubes greatly influence the biodegradable process. In fact, the biodegradability starts at the surface of PLGA nanoparticles and creates the place for cell growth. The degradation process gives rise to a fibrillar structure in which CNTs form a framework-like arrangement, Fig. 4.7-1, that overwhelms the releasing effects of nanotubes, a

critical point for long term biocompatibility. The permanence of nanotubes in a structured network increases the contact with cells and maintains their biofunctionality during and after the biodegradation process of macroscopic fiber.

This approach results in biocompatible fibers, able to stimulate the growth of nerve cells. Here we present results of high cell viability for PC12 cells which prove the ability of CNT- fibers to influence the growth of nerve cells, Fig. 4.7-3 The presence of nanotubes encourages the cell proliferation; significative difference (p<0,05) has been determined between different fibers and control. Moreover, the hybrid approach greatly improves the CNTs' distribution in fiber, thus enhancing the biocompatibility of material and their capacity to interact with cells. This multifaceted functionality of the hybrid fibers is attributed to the intrinsic characteristics of CNTs combined with particular properties of PLGA nanoparticles. A strong relationship exists between the nanotubes' organisation and the cells' morphogenesis. Indeed, cell adhesion is related to the presence of nanotubes onto the surface, Fig. 4.7-2a,2b. With a such distribution, the nanotubes, possessing a high specific area, become a sensitive means of stimulating cell growth while improving their morphogenesis, Fig. 4.8-1. Furthermore, they incite the differentiation of cells, expressed by the formation of neurites and their extension, Fig. 4.8.-2. Even in the absence of nerve Growth Factors (NGFs), a stimulator for the differentiation of cells, the fibrous substrate promotes and sustains the formation of neurites onto the fiber, Fig. 4.8-1, as well as in its environment, Fig. 4.9-2. Endowed with immanent characteristics, these new fibers are designed as a new substrate for neural cells, capable not only of sustaining their proliferation but also of guiding nerve

growth in a preferentially controlled way, Fig. 4.7-2a, 2c. The textured surface with a periodic columnar order, Fig. 4.6-2, and particular topological features, Fig. 4.6-1, enhances the fiber contact with cells, Fig.4.8-2, and favours their interactions [38].

The textured surface observed by electronic microscopy, Fig. 4.6-2, demonstrates that each fiber possesses a particular nanotubes' network, tailored by the formulation, that endow it with specificity in the contact with cells. The fiber's structural organization in a branched-lengthened form, Fig. 4.6.-1, at nano-, micro- and macro- scales, is similar to the fibrillar structure of the extracellular matrix and imitates the network of neurons' organization, Fig. 4.1-2a. This structuration allows the interconnection between neurites which follow the direction of nanotubes and the fibrils including them, Fig. 5.8-2b. The neurites extend in the direction of the aligned nanotubes.

The results not reported here prove the good mechanical performance of the hybrid fibers. Indeed, the structural characteristics determine the enhancement of mechanical properties, particularly their elasticity and flexibility which have consequences on the interactions with nerve cells [11,39]. Flexible fibers establish a proper contact with neural cells, Fig. 4.9-1. A right balance between the fiber's structure, its surface topography, mechanics and composition exerts a beneficial effect in the interaction of fibers with neuronal cells. This synergistic action controls the cellular response through the cell-substrate interface.

We created new CNT fibers as a promising substrate for stimulating the growth of neuronal cells. We particularly envision the use of these hybrid biomaterials for the

restoration of injured spinal cords where the fiber's elasticity and flexibility are important assets.

4.5 METHODS

SWNTs were purchased from Carbon Nanotechnologies Inc. and Multi-Wall Carbon Nanotubes from Arkema. Mixture containing 0.3 wt.% SWNTs, 1.0 wt.% SDS and 98.7 wt.% water, was ultrasonicated using a sonicator horn during 60-80 min at 40KW to obtain D-SW. Mixture containing 0.9 wt.% MWNTs, SDS 1.2 wt.% and 97.9 wt.% water was ultrasonicated with a sonicator horn during 45-60 min at 20KW to obtain D-MW.

Two PLGA copolymers with 50:50 ratio (lactic: glycolic acids) were purchased from Boehringer Ingelheim: Resomer RG 502 (RG 502), viscosity of 0.16-0.24 dlg^{-1}, MW of 12 KDa and Resomer RG 503H (RG503H) viscosity of 0.32-0.44 dlg^{-1}, MW of 34 KDa. Two solutions were prepared for each Resomer: 1) 200 mg were dissolved in 13 mL acetone; 2) 600 mg were dissolved in 13 mL acetone. 12 mL distilled water were added dropwise in each solution for nanoprecipitation. The blends were stirred at room temperature until complete evaporation of acetone to yield 12,5 mL aqueous suspensions:, S1-RG502, S2-RG502, S1-RG503H, S2-RG503H.

2 mL of S1-RG502 and S2-RG502 were added to 1 mL SW dispersion to obtain 3 ml of

M1-SW- RG502 and M2-SW-RG502 mixture. 3 mL of S1-RG503H, S2-RG503H were added to 1 mL SW dispersion to obtain 3 ml of M1-SW-RG503H and M2-SW-RG503H mixture. 7 mL of S1 and S2-RG502 were added to 1 mL D-MW to obtain 8 ml of M1-MW-RG502 and M2-MW-RG502 mixture. 5 mL of RG503H-1, RG503H-2 were added to 1 mL MW dispersion to obtain 6 ml of M1-MW-RG503H and M2-MW-RG503H mixture. The mixtures were sonicated 15 min for homogenization.

88-89% hydrolysed PVA powder with MW of 15KDa was purchased from Sigma-Aldrich. 50g PVA was dissolved in 950 mL distilled water, at 95°C, during 45 min, while stirring, then the 5% PVA aqueous solution is cooled at room temperature.

Spinning protocol was conducted following the experimental techniques, Chapter 3.

Measurement of Diameter of Nanospheres: The mean diameter of PLGA nanoparticles and the polydisperity index (PI) of the distribution were determined by Dynamic Light Scattering (DLS) using a Zetasizer Nanoseries (Malvern instrument, UK).

Scanning Electron Microscopy (SEM) was performed using a Hitachi System, Model S-3550N, The samples were no coated with a conducting layer, thus preserving their features.

AFM imaging was performed using a Nanoscope III A controller, a multimode AFM head (Digital Instrument Santa Barbara CA, USA) with a silicon cantilever in taping

mode. AFM amplitude, topography, and phase scans were recorded on a fiber area of $500 \times 500 \, nm^2$, $5 \times 5 \, \mu m^2$ and $10 \times 10 \, \mu m^2$.

Thermo-gravimetric analysis (TGA) was performed to determine the CNT content using a Setaram instrument, by heating from 25 to 600°C, at a rate of 10°C/min, under argon atmosphere.

Biodegradability was assessed over a 6 week period in phosphor buffer saline (PBS), at 37 ° C, then the fiber morphology and topology were investigated using SEM.

Biocompatibility assay was performed using PC12 cells. We investigated the fibers ability to stimulate the proliferation of nerve cells and to contribute to the extension of neurites, as an indicator for cell differentiation. Three aspects were studied: proliferation, adhesion, and differentiation of the PC12 cells. These cells can cease proliferating and undergo differentiation in the presence of neural growth factors (NGFs). 20 fibers were disposed onto a pre-cleaned glass slide (7×7 mm^2) to prepare a sample. Rat PC12 cells, obtained from American Type Tissue Collection, and were used in the cell cultures. Type II collagen, purchased from Sigma-Aldrich Canada Ltd., was used to coat the plates to improve the adhesion. RPMI Medium 1640, containing 10% heat-inactivated horse serum and 5% fetal bovine serum, was purchased from Gibco-BRL and used as growth medium. PBS was purchased from Sigma-Aldrich Canada Ltd. The culture medium was supplemented with 50ng/mL Neural Growth factors (NGF- Gibco–BRL, NY, USA) to

induce cell differentiation. The control sample was a collagen coated 7x7 mm^2 glass slides.

The MTT metabolic assay was carried out following the standard procedure to study the cellular growth through changes in cell proliferation, viability and cytotoxicity. The results were read on a multiwell scanning spectrophotometer (ELISA reader); a relationship between cell number and absorbance was established through the variation of absorbance (Optical Density, OD).

Optical microscopy (OM) was carried out using a Nikon Eclipse E800 microscope, equipped with a digital camera, to daily observe the fibers in cell culture.

Scanning Electron Microscopy (SEM) was used for the examination of samples after three days of culture. The cells fixed with 4% paraformaldehyde in PBS for 15 min were washed with distilled water and gradually dehydrated in ethanol.

Fluorescence microscopy (FM) was performed with an epifluorescence microscope (Axiophot, Zeiss). Hoechst staining was used to identify the cells attached to the membrane as well as to identify the formation of neurites. The samples were then incubated at room temperature for 15 min in 2 mL of 2μg/mL Hoechst solution.

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LEGEND OF FIGURES

Figure 4.1 **Colloidal Mixtures and their Corresponding Fibers. 1-a,b,c,d.** SEM micrographs reveal the homogeneous distribution of four different colloidal mixtures : M1-SW-RG503H, M1-SW-RG502, M1-MW-RG502, M2-MW-RG503H; the arrows indicate this distribution. **2-a,b,c,d.** Cross sectional views of the corresponding fibers: SW-RG503H-1, SW-RG502-1, MW-RG502-1, MW-RG503H-2 suggesting the role of the mixture in the construction of the fiber's internal structure. **3-a,b,c,d.** Micrographs depicting the geometry and the topography of SW-RG503H-1, SW-RG502-1, MW-RG502-1, MW-RG503H-2 fibers at macro, micro and nano-level. The nanosized exotic texture of these fibers, indicated by arrows, confirms the efficiency of the hybrid approach.

Figure 4.2 **Homogeneous Phase Distribution at the Surface of CNT Fibers. 1-a,b,c.** Taping mode AFM phase images of various scan areas, taken along the fibers: SW-RG503H-1, SWRG502-2, MW-RG502-1 show the formation of the specific domains homogeneously distributed at the surface. SW-RG503H-1 microscopic surface **(a)** shows interconnected PLGA nanoparticles, indicated by arrows while the SWRG502-2, MW-RG502-1 fibers surface **(b,c)** is marked by the presence of orientated fibrilar domains with the width ranging from 15 to 25 nm for SWRG502-2

fibers and from 20 to 40 nm for MW-RG502-1 fibers. **2-a,b,c.** Taping mode AFM phase images taken perpendicular to the fiber's axis confirm the similarly ordered structure with specific details. The SWRG502-1 fiber surface (**a**) is characterised by the ordered spherical nanoparticles while the presence of worm-like domains characterises the MW-RG503H-1 surface (**b**). MW-RG503H-2 fiber displays a lamellar structure (**c**).

Figure 4.3 **Contribution of Colloidal Mixture to the Fiber's Structure. 1.** Micrographs revealing the homogenous films of M1-SW-RG502 and M2-SW-RG503H mixtures and the formation of the pearl-like chains. **2.** Cross section of the corresponding fibers: SW-RG502-1 and SW-RG503H-2, as viewed with SEM. The evidenced pearl-like chain arrangements, as indicated by the arrows, (**1-a,b**) result in very particular internal structures (**2-a, b**).

Figure 4.4 **Development of the Nanotube-Sphere Binary Colloidal System (NSBCS). 1.** Preparation of an aqueous dispersion of CNTs using an appropriate surfactant, sodium dodecyl sulphate (SDS). **2.** Preparation of the PLGA aqueous suspension through the nanoprecipitation method. In the absence of a surfactant, the suspension is quasi-stable and contains nanospheres with a variable diameter depending on the PLGA characteristics. **3.** Preparation of a colloidal mixture as a Nanotube-Sphere

Binary Colloidal System (NSBCS) by blending different ratios of CNT dispersion and PLGA suspension. **4.** View on the organisation of the sphere-like particles with the rod-like particles in a homogenous mixture. The schemas are not based on a scale proportion.

Figure 4.5 **Confinement and Coagulation in the Spinning Process. a.** Schema depicting the confinement during the injection of a CNT-PLGA mixture in the coagulating bath: **(1)** A colloidal mixture placed in the cylindrical chamber of the syringe where nanotubes are inserted in the gaps of copolymer nanospheres lattice. **(2)** A colloidal mixture submitted to the spatial confinement imposed by the passage from the syring' needle to the conical nozzle and throughout tit, where the proto-fibers are formed as the precursors of the fibers. **b.** Coagulation process takes place at the contact of the proto-fiber with the polymer chains of the coagulating bath. PVA is partially adsorbed onto the nanoparticles' surface while other chains will initiate the bridging flocculation including both rod-like and sphere-like particles. **c.** SEM images of a fiber containing CNT reveal its textural features and structural particularities.

Figure 4.6 **Fiber's Morphology and Texture. 1-a,b,c,d.** Topographic AFM images, in taping mode, taken along the fibers: SW-RG503H-2, SW-RG502-1, MW-RG503H-1, MW-RG502-1. More detailed examination of

morphology reveals the morphological diversity, at nano- and micro-scale, which could be created using SWNTs and MWNTs combined with PLGA nanospheres. **2-a,b,c,d.** SEM images of SW-RG503H-2, SW-RG502-1, MW-RG503H-1, MW-RG502-1 fibers depict their texture and surface morphological features.

Figure 4.7   **Biodegradability and Biocompatibility of CNT-based Fibers. 1-a,b.** SEM micrographs illustrate the surface of a CNT-based fiber following a 6 week incubation period in PBS solution; the biodegradation is revealed by the change of the texture and the emergence of nanotubes from the fiber surface, as indicated by the arrows. **2-a,b,c.** SEM micrographs illustrating PC12 cells grown on SW-R503H-1 fibers following a 3 day culture period, in the absence NGFs; the arrows show the cells' spreading in the fiber's direction (**a**) in addition to their adhesion onto the fiber (**b**) and a view of the PC12 cells with their morphological characteristics (**c**). **3.** A diagram illustrating the variation of PC12 cell viability cultured onto the fibers with different CNT content and comparaison with a positive control whose the content in nanotube is zero.. The optical density, OD, was measured with the ELISA plate reader at 570 nm; the absorbance was proportional to the number of living cells present, as an expression of cell viability after a three day culture period. The OD value is influenced by the presence of CNTs into the fibers as suggested by the comparaison with the control. Each bar

color represents one type of fiber; pink designates SW-based fibers while blue indicates MW-based fibers The control is represented by the violet color.

Figure 4.8 **Growth of PC12 Cells and Formation of Neurites onto the CNT Fiber Substrate. 1-a,b.** FM images show PC12 cells cultured on SW-RG502-2 and MW-RG502-1 fibers, following a 3 day culture period in the absence of NGFs. **1-c.** FM image of PC12 cells cultured on SW-RG502-1 fiber; in the presence of NGFs, the cells grow and align alongside the fiber and form neurites which extend along the fiber, as indicated by the arrows. **1-d.** FM image of PC12 cells cultured on MW-RG502-1 fibers, following a 3 day culture period in the presence of NGFs, confirms the growth of cells which surround the fiber and the formation of neurites and their extension. Original magnification for 1-a,b,c,d: x100. **2-a.** SEM micrograph shows the adhesion PC12 cells on the SW-RG503H-1 fiber whose growth is directed by the aligned nanotubes. **2-b.** In the absence of NGFs, the PC12 cells grown onto the SW-RG503H-1 fiber extend their neurites which interconnect with others to form a network.

Figure 4.9 **PC12 Cells in Contact with Flexible Fibers. 1-a,b.** FM images of PC12 cells cultured onto SW-RG503H-1 and MW-RG503H-2 fibers, in the absence of NGFs, following a 3 day culture period. We visualise, as

indicated by the arrows, the fiber's flexibility in relation with the formation of neurites, their extension and their alignment. **2-a,b**. OM images of PC12 cells in contact with MW-RG503H-1 fibers in the presence of NGFs. Original magnification: x100. First image (**a**) taken with an optical microscope, following a 2 day culture period, exemplifies cells alignment alongside of fibers and the second (**b**) illustrates the formation of neurites on the SW-RG503H-1 fiber and in its surrounding, following a three 3 day culture period. Original magnification: x200.

LEGEND OF TABLES

Table 4.1

Content of CNTs and Polymer Components as Determined by TGA Measurments.

Table 4.2

Size of PLGA Nanoparticles as Measured by DLS and AFM Techniques.

FIGURES

1 2 3

a

b

c

d
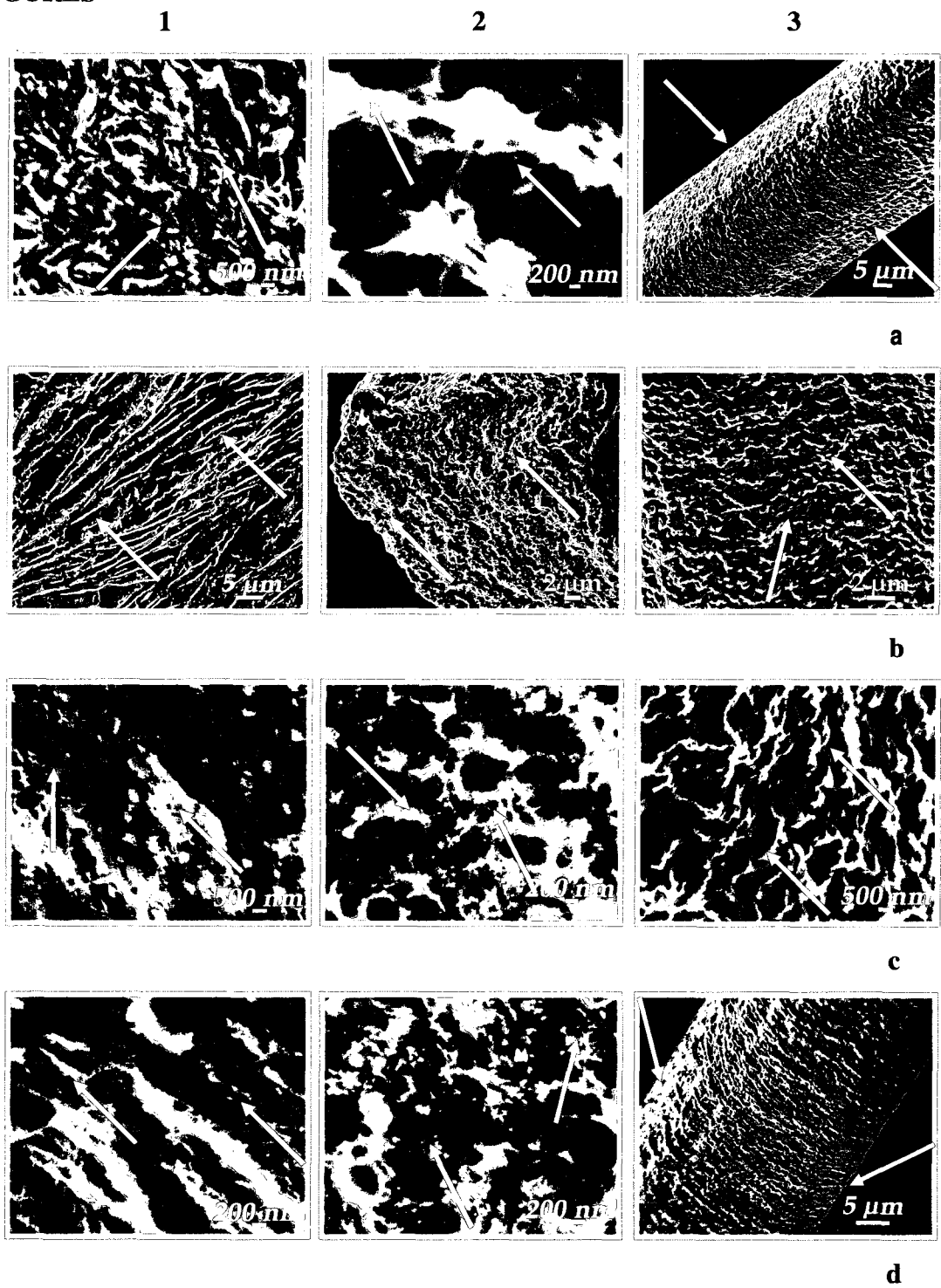
Figure 4.1

Figure 4.2

Figure 4.3

a

CNT pristine + SDS surfactant → CNT dispersion

b

acetone

PLGA solution

water

PLGA powder

NANOPRECIPITATION

evaporation

PLGA aqueous suspension

PLGA nanoparticles

CNT
dispersion

PLGA
nanoparticles

Colloidal
Mixture
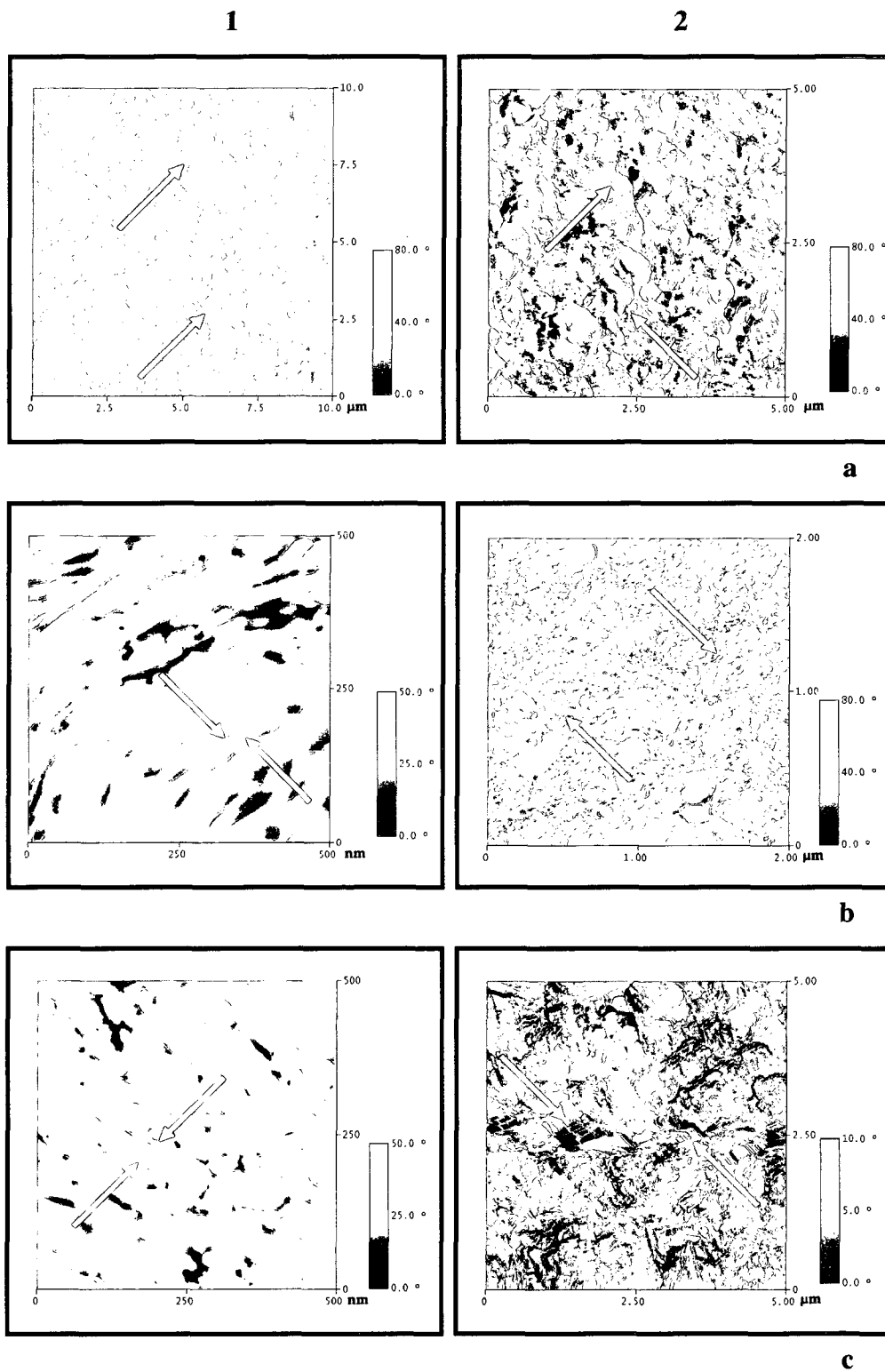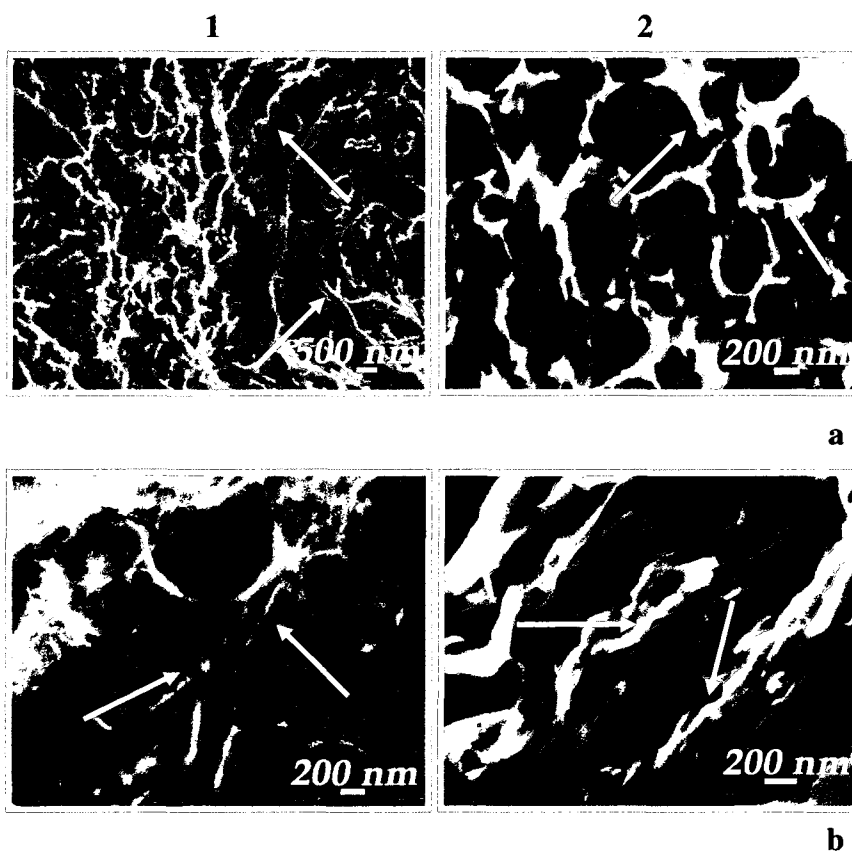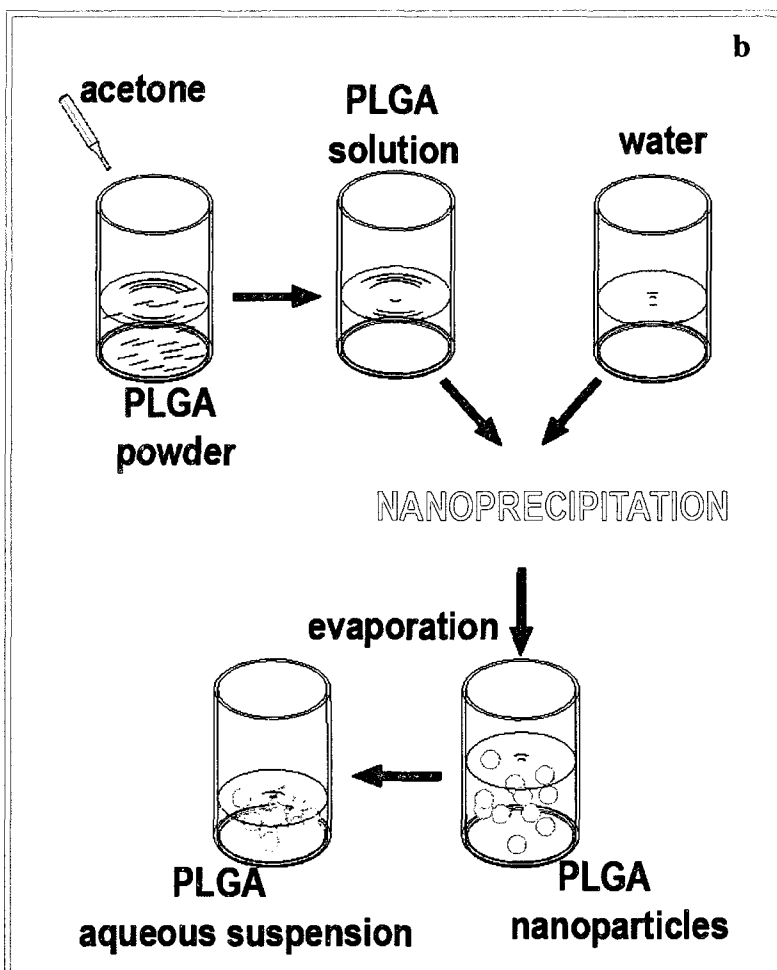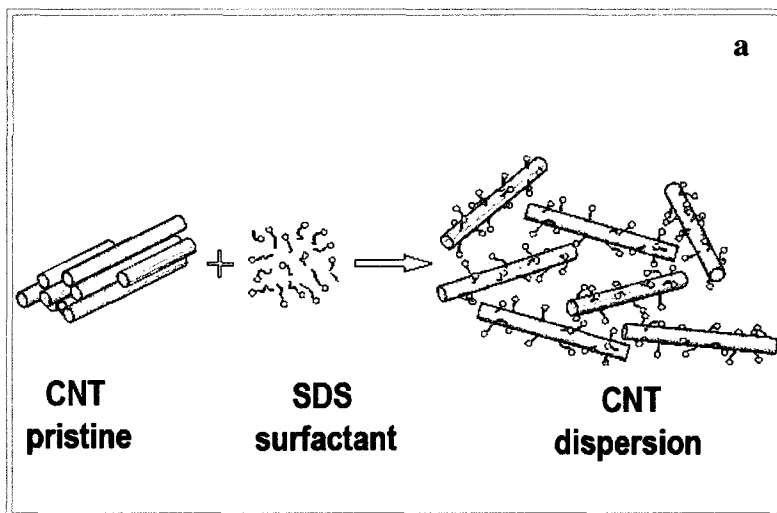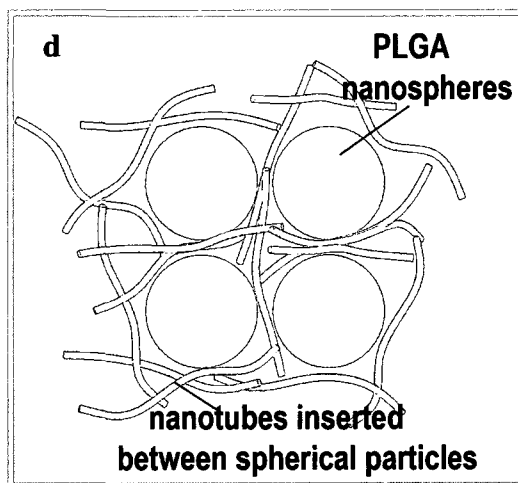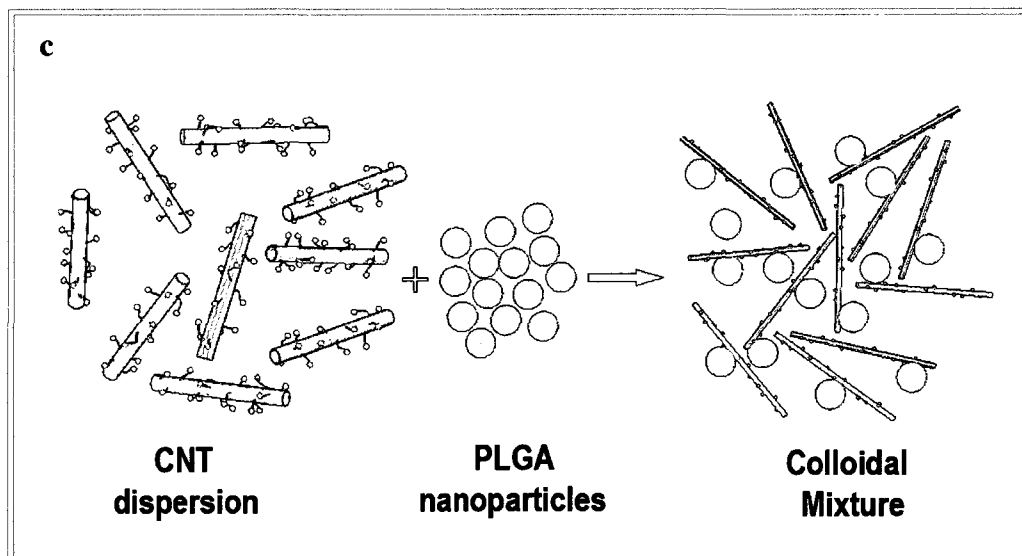
Figure 4.4

Figure 4.5

1

2
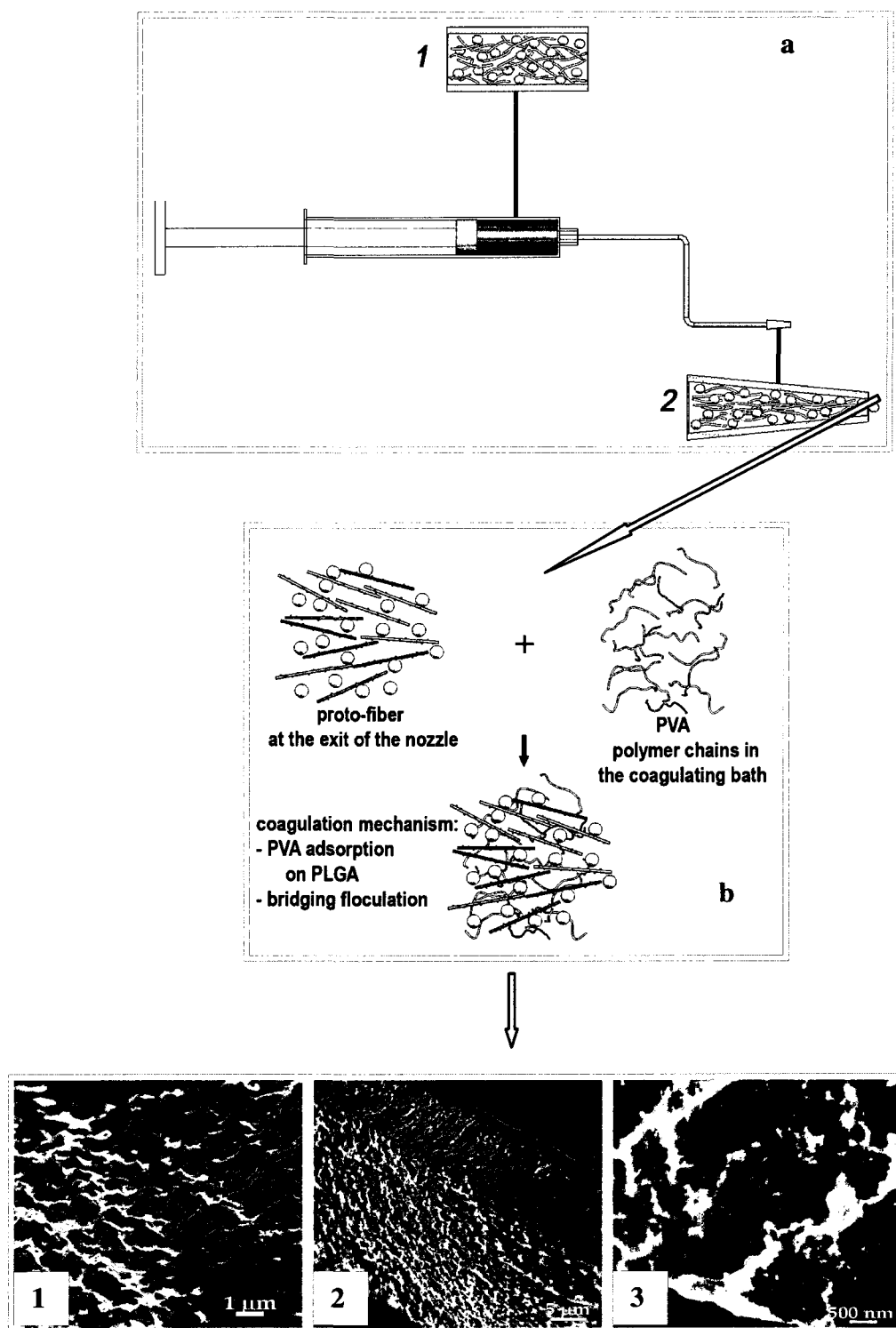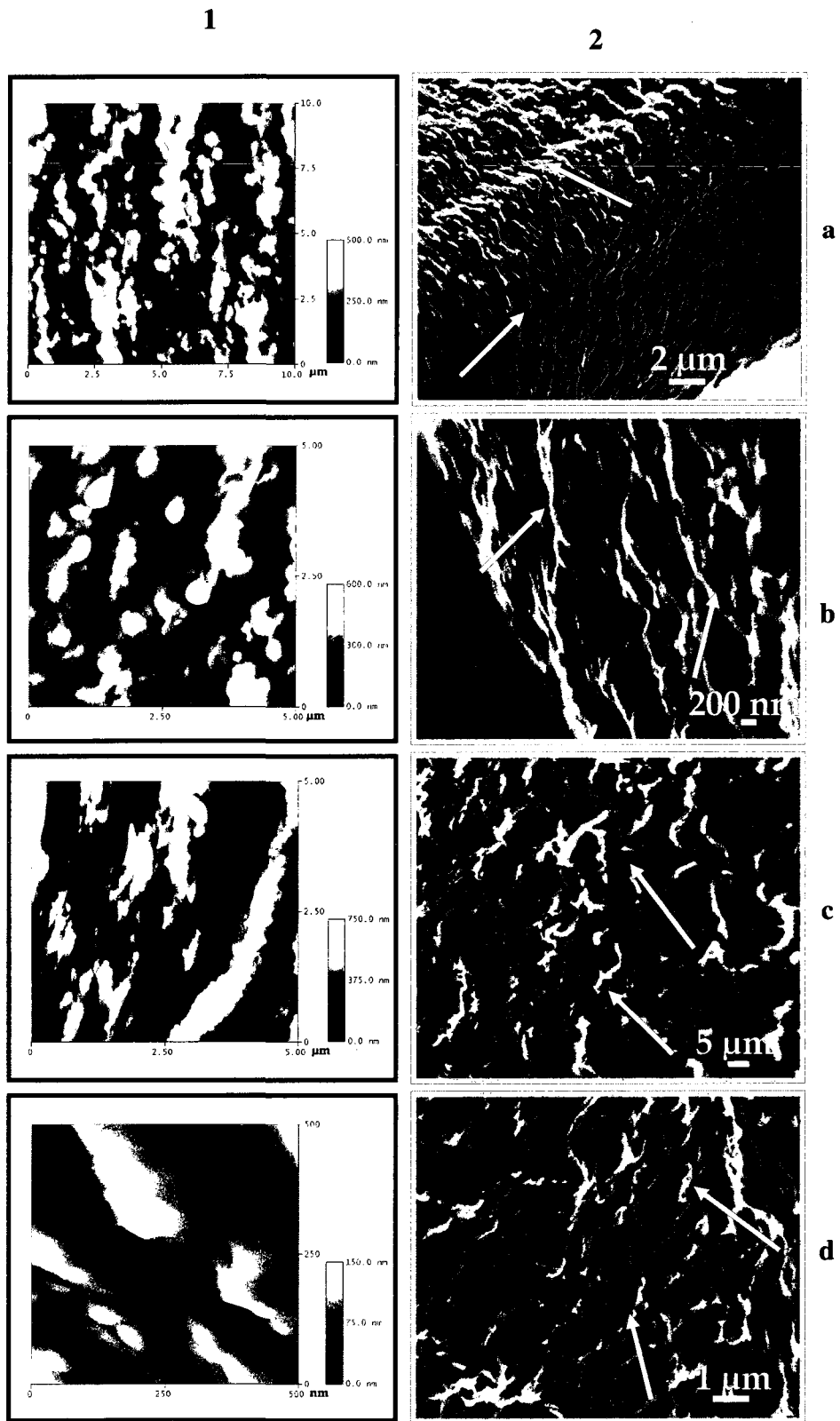
Figure 4.6

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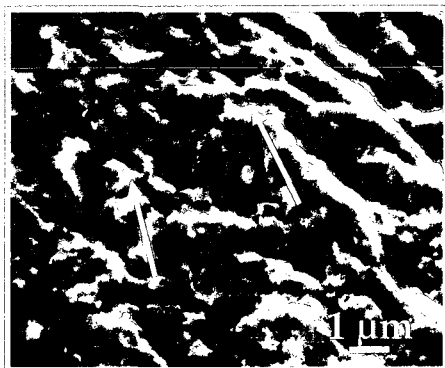
a

b

2

a

b

c

d

3

Figure 4.7

1

d

2

2 μm   1 μm

a   b

Figure 4.8

1

a   b

2

a   b
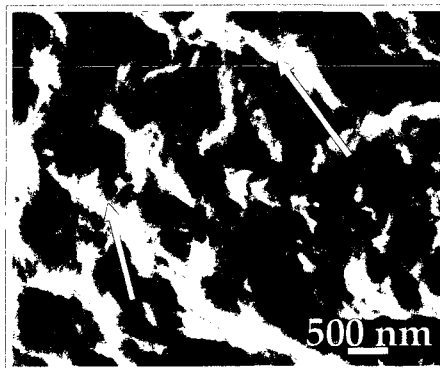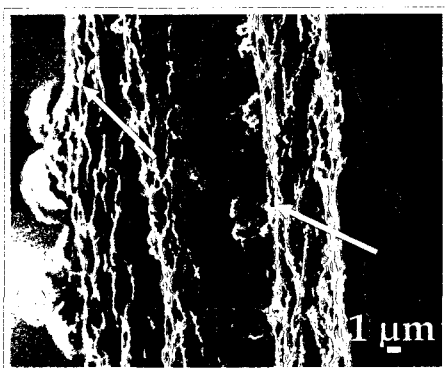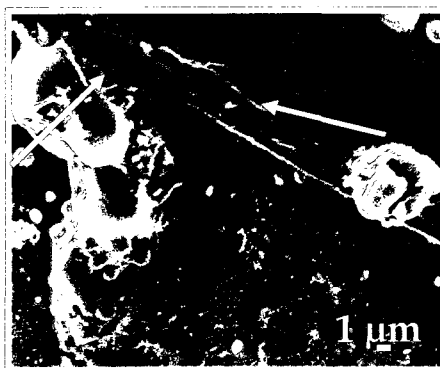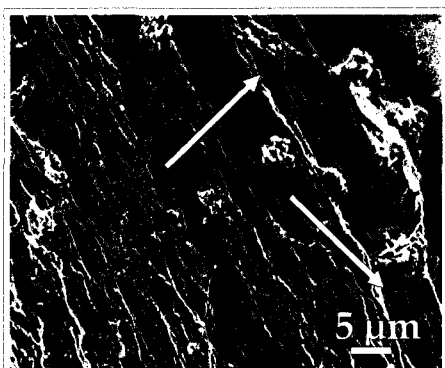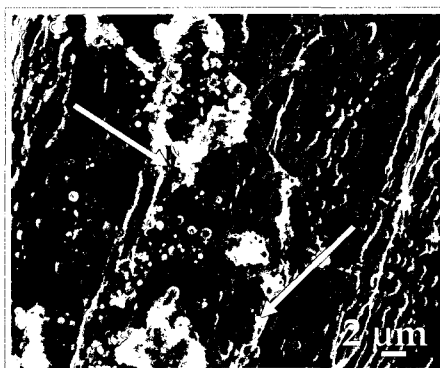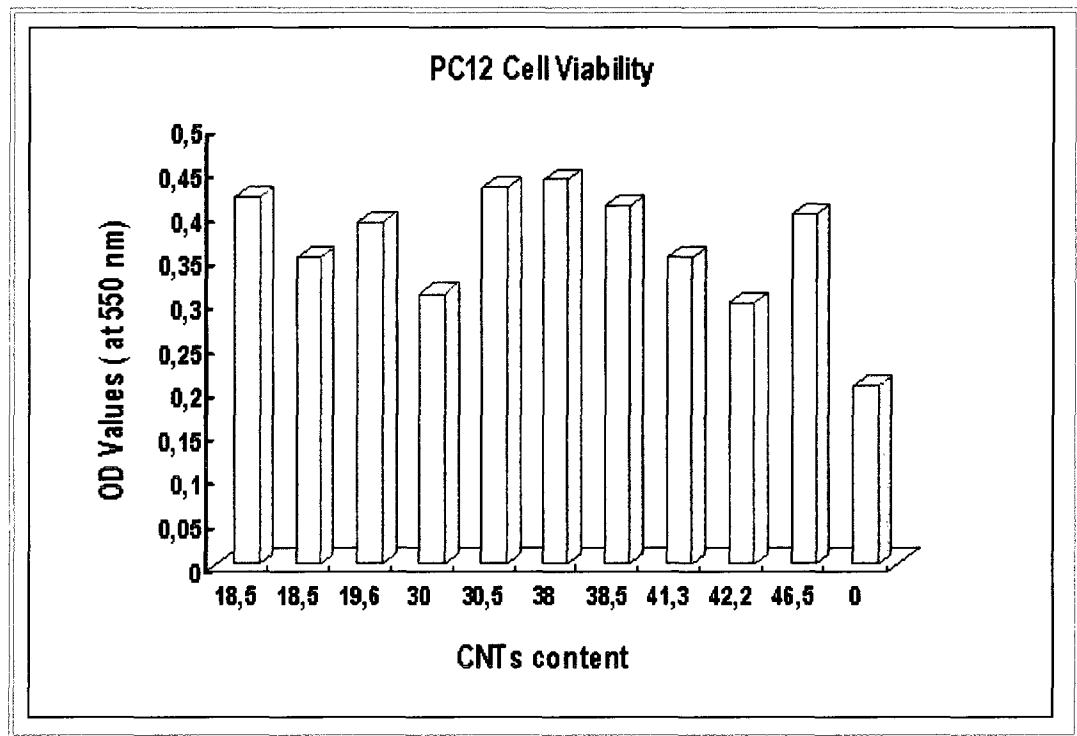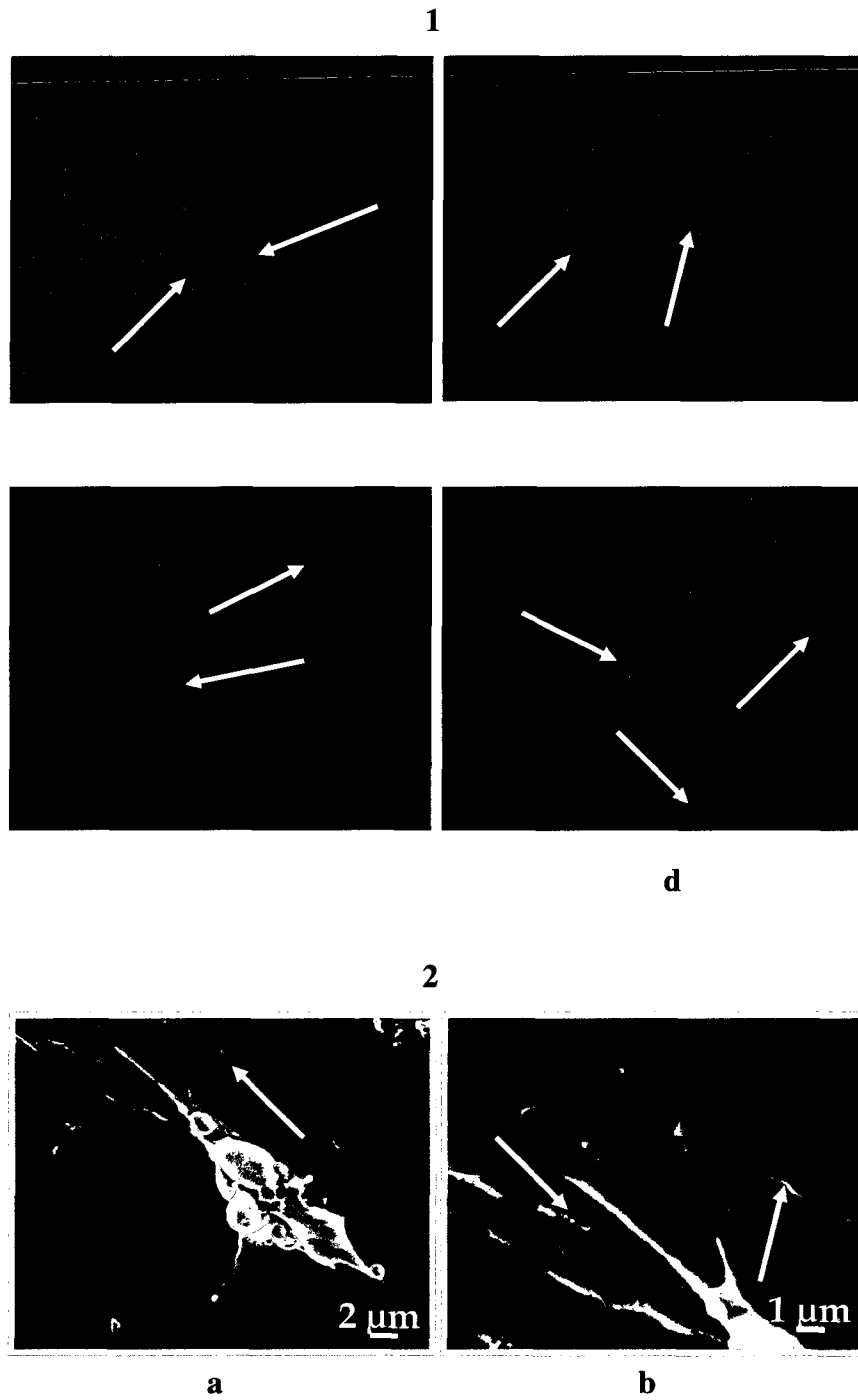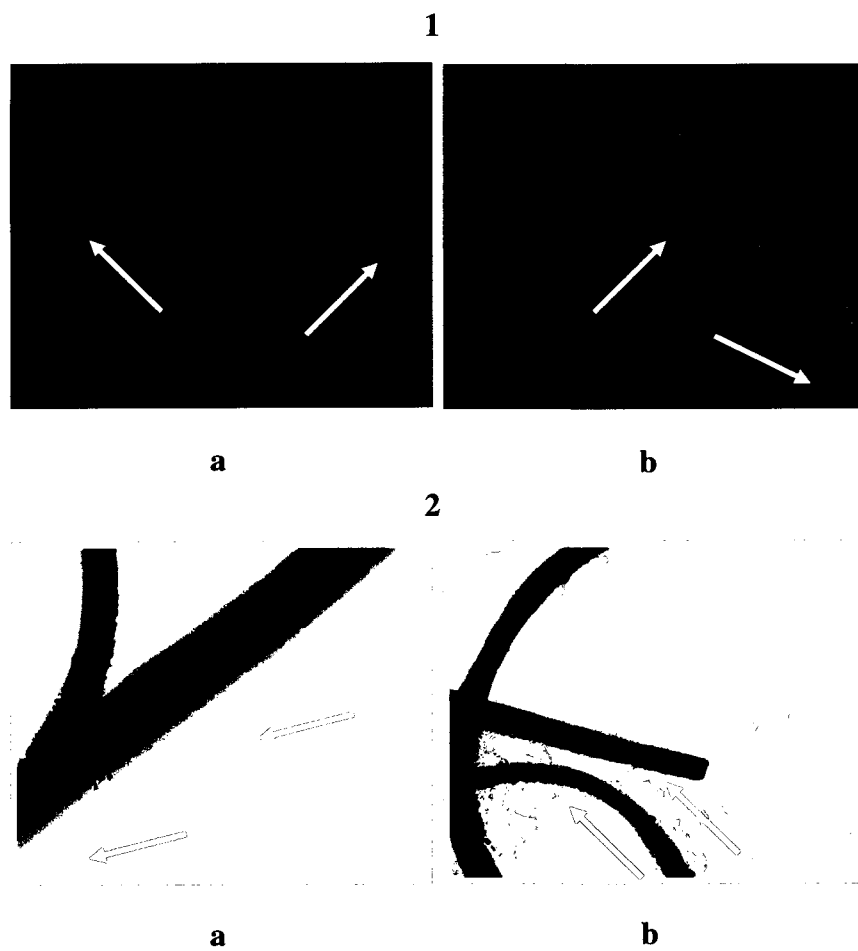
Figure 4.9

TABLES

Table 4.1.

Fiber Sample	Fiber Description	Component Content	
		Polymer (%)	CNT (%)
SW-RG503H-1	SWNT, RG 503H-1, PVA	81.5	18.5
SW-RG503H-2	SWNT, RG 503H-2, PVA	80.4	19.6
F-SW-RG502-1	SWNT, RG 502-1, PVA	81.5	18.5
F-SW-RG502-2	SWNT, RG 502-2, PVA	70.0	30.0
F-MW-RG503H-1	MWNT, RG 503H-1, PVA	69.5	30.5
F-MWR503H-2	MWNT, RG 503H-2, PVA	61.5	38.5
F-MW-RG502-1	MWNT, RG 502-1, PVA	62.0	38.0
F-MW-RG502-2	MWNT, RG 502, PVA	53.5	46.5

Table 4.2.

PLGA Nanoparticles in Aqueous Suspension	Mean Diameter of nanoparticles measured by DLS nm	PI of the distribution of nanoparticles	Size of nanoparticles determined from AFM images nm
S1-RG503H-1	160	0,075	170
S2-RG503H-2	180	0.085	180
S1-RG502-1	90	0,183	100
S2-RG502-2	100	0,098	110

CHAPTER 5

FIBRILLAR STRUCTURES SUPPORTING

THE GROWTHOF LIVING CELLS:

HYBRID INTEGRATION OF SWNTs IN MACROSCOPIC FIBERS

AND THEIR CHARACTERISTICS AT NANOSCOPIC LEVEL

Fibrillar Structures Supporting the Growth of Living Cells:

Hybrid Integration of SWNTs in Macroscopic Fibers and their

Characteristics at Nanoscopic Level

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5.1 ABSTRACT

In this paper we present the distinctive characteristics of SWNT-based fibers designed as biomaterials. Our investigation concerns the SWNTs-based fibers in terms of structure, morphology and mechanics. A relationship between structure, morphology and mechanical characteristics is established in order to confirm the efficiency of this hybrid approach in the integration of the nanoscale characteristics of nanotubes into a macroscopic shape. We also demonstrate how these characteristics are reflected in the function of biomaterials when in contact with living cells.

5.2 BACKGROUND

In the human body cells grow on 3-D fibrillar structures, such as extracellular matrix (ECM). Shaping the substrate for cell culture in a fiber form is an advantage for cell and tissue engineering. Fiber's ability to orient cells and encourage their motion has been demonstrated [1] before. Indeed, if fibers cross each other, cells may bridge from fiber to fiber forming a sail structure [2]. This initiated a correlation between the substrate shape and cell's evolution when in contact with fibrillar substrate. Employing macroscopic fibers for the construction of 3-dimensional supports is a convenient option using the classical textile processes while maximizing the advantages of fiber shape in their relation with living cells. Furthermore, the development of nanotechnology and the emergence of nanostructures provide new supports for cells. Among them, polymeric nanofibers and carbon nanotubes (CNTs) are promising candidates for these applications [3,4] .

The role of carbon nanotube structure in relation with the growth of fibroblasts, as main component of ECM, has been studied. The investigation of fibroblasts cultured onto MWNTs aligned by dielectrophoresis evidenced a correlation between cell growth, morphology and alignment of cells [5]. Cell extensions have been identified as the primary attachment mechanism with a hairy appearance complemented by a morphology that resembles that of fibroblasts found in native tissue. The electrospun nanofibrous scaffolds containing multi wall carbon nanotubes (MWNTs) and polyurethane (PU) have been considered [6]. The results proved that these architectures provided a suitable

environment for cell migration and contact with the microenvironment, thus enhancing cell adhesion, proliferation and migration. In this work the nanotubes did not exhibit cytotoxic activity against, 3T3-L1 mouse fibroblasts, thus proving the efficiency of CNT-composites for such a purpose. In addition, the construction of a 3–dimensional MWCNT-based network has been evidenced as ideal candidate for scaffolds in tissue engineering. The mouse fibroblast cultured on this construction confirmed the extension of cell growth, their spreading and adhesion [7].

With the progress of nanotechnology, nanoscale topography becomes a tool in the development of materials possessing the ability to control cell adhesion and response [8]. Previous works demonstrated the importance of mimicking the nanotopology of the ECM [1,2,3]. Moreover, the study of nanofibers and their interactions with fibroblasts demonstrated the determinant role of nanofiber diameter in the adhesion of fibroblasts. Very recent work investigated the cytotoxicity of SWNTs in relation to human fibroblasts and proved that the refined SWNTs are more toxic than unrefined SWNTs, thus suggesting the role of surface area and surface chemistry as variables of the toxic effect [9].

Due to their outstanding characteristics, CNTs hold great promise in the construction of nanosized substrate for cell growth and their proliferation, however they are limited in terms of water solubility, and biodegradability [10,11]. Adapted wet spinning process provides an attractive alternative method for the production of CNT-based fibers [12,13]. We developed a new method for the production of biodegradable

fibers containing carbon nanotubes [14] with a biocompatible response. Through this method we preserve the chemical inertness of nanotubes and allow a homogenous exposure of their high surface area in a framework with polymeric nanoparticles. This geometric alternation determines the structuring of fibers, their texture and further creates a favourable contact with cells, thus positively affecting their growth.

In this work we describe three main particularities of SWNT-based fibers designed as substrates for fibroblasts. They resulted from the integration of single wall carbon nanotubes (SWCNTs) at nanometric level, in multi-scale architecture fibers. The first focuses on nanoscale structural aspects while the second points to surface morphology. The third aspect originates from internal organisation of the hybrid fibers and includes mechanical characteristics and viscoelastic behaviour. The latter, as sensitive indicator of internal structure reveals a structure-property relationship of fibers. Four different samples were cultured with human skin fibroblasts in order to evaluate their biocompatibility as a growing support for human skin fibroblasts. The four samples with various SWNT content have been investigated in relation to these cells. The efficiency of the SWNT-based fibers cultured with fibroblasts is presented in terms of proliferation, adhesion and migration.

5.3 INTEGRATION OF CNTs AND FIBERS' CHARACTERISTICS

The macroscopic fibers we investigate were produced by a new spinning method [12] consisting in the injection of a colloidal mixture in a poly-vinyl alcohol (PVA)

solution as coagulating agent. This mixture results from blending the aqueous dispersion containing SWNTs and sodium dodecyl sulphate (SDS) with an aqueous suspension of poly-lactic-co-glycolic acid copolymer (PLGA) nanospheres. We thus assembled several hundreds of centimetres long fiber material containing SWNTs, PLGA and PVA. These fibers have a circular shape and show very homogenous internal structure consisting of SWNT thin bundles interlaced with PLGA nanospheres, Fig.5.1-1. The integration of SWNTs in the macroscopic form follows a supramolecular organization, a process intermediated by PLGA spherical particles which trigger this organization and promote the creation of fibrillar constituents. Through a structural and morphological evaluation, we provide the specific characteristics of fibers produced by this hybrid approach combining nanotube cylindrical molecules and the spherical shape of PLGA nanoparticles. We thus emphasize the determinant role of nanoscale integration of nanotubes in a tertiary blend. In fact, we are interested to obtain fibers that are able to preserve the SWNTs characteristics while offering maximum exposure of their high specific area. The fiber bulk composition, as determined by thermal gravimetric analysis (TGA), and presented in Fig. 5.2, demonstrates the variability of composition conferred by different formulations using PLGA copolymer. Three samples have similar SWNT content while in the fourth sample this content increases with 65%. We are interested to determine the main structural characteristics of fibers in relation to the nanotubes fraction they contain.

Through this formulation the PLGA nanospheres are introduced in macroscopic fibers in a non-covalent way with beneficial effects measured at the molecular level. The

reversible contacts between nanotubes and nanospheres, possibly supported by their hydrophobic interactions, promote the association nanotube-nanosphere that extends into fibrillar structure, Fig. 5.1-2.

The microscopic observations using the Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) demonstrate the variation of structural and morphological characteristics at microscopic and submicroscopic level. Partially joined to nanoparticles, SWNTs form connection points with them, thus creating the constitutive elements of internal structure, Fig. 5.1-1. Together, these assemblies form a monofilament thread built from multiple fibrillar constituents, Fig. 5.1-2, including SWNTs, PLGA copolymer and PVA polymer. The specific organisation of these elemental arrangements at nanoscopic level determines the texture of fiber, Fig. 5.3-a,b,c,d.

By AFM examination we identified various topographic dissimilarities between the four samples, for both the longitudinal and the lateral area. The morphological features depicted by AFM imaging, in tapping mode, suggest the formation of well organised fiber characterized by a specific configuration, Fig. 5.1-2d. The profile of each fiber is described by the periodic variation of its main feature, thus generating a rough surface, Table 5.1. We choose the values of root mean squared (RMS), a deviation of the peaks and valleys from the mean plan, to express the roughness of fibers. The RMS measurements were performed for three different areas along fiber and across fiber: $10 \times 10\mu$, $5 \times 5\mu$ and 500×500 nm. The results suggest a correlation between these values and the characteristics of PLGA nanoparticles at different levels. Indeed, the large dimater particles contained in the S1-RG503H-1 suspension, Table 4.2, provide the higher value of roughness, Table 5.1,

alongside of fiber. At the same time, the roughness across fiber is rather influenced by the tendency of nanospheres to agglomerate; the highest value of PI of S1-RG502 nanoparticles, Table 4.2, results in the high rough surface of SW-RG502 fibers, Table 5.1

The maximum integration of nanotubes in the host polymer engages the development of a strong interface between the various components providing the topological stability to the nanotubes in the process. The orientation of nanotubes along the fiber axis plays an essential role in the enhancement of elastic modulus of macroscopic fibers while a strong nanotube-polymer interface strengthens the fibers. The mechanical behavior of CNT-based fibers was investigated using an Instron 4301 testing machine in tensile mode. The stress (σ), strain (ε) and Young's modulus (E) were determined for each type of fiber using the curve stress-strain that shows the resistance of fiber upon stress and its deformation. The values of the elastic modulus were determined from the linear part of this curve. The results of mechanical characteristics are presented in Table 5.2. For the fiber containing 19% SWNTs we report an average values of 140 MPa for tensile stress, the elastics modulus values ranging from 12 GPa to 20 GPa and a deformation of 2% to 5%. An example is presented in Fig. 5.4. The measured mechanical characteristics are correlated to the microscopic observations performed on samples resulting from mechanical testing. We are thus able to establish a relationship between structure, morphology and mechanical behaviour. In fact, we attribute the variability of stress values to the copolymer which varies in molecular weight and concentration. In addition, the calculated elastic modulus of 18 GPa, is a good indicator of the achievement of the strong interface between

nanoparticles and nanotubes, as depicted in the SEM micrographs taken on the sample after they have been tested, Fig.5.5-1, In the case of the fiber containing 30% SWNTs, the stress and elastic modulus values are slightly reduced. We identified a particular response from the sample SW-RG503H-2, which displays a deformation of 30%. This behavior is not completely understood and we speculate that it originates from the arrangement of nanotubes between the nanospheres and their association in the formation of the filament. A more relaxed internal structure could be observed in the micrograph of tested samples Fig.5.5-1d. The pullout of individual nanotubes suggests a loose interface between nanotubes and spheres with an effect on fiber resistance, when it is submitted to the load. However, the decrease of elastic modulus is not severe. A good elastic modulus and mechanical resistance combined with a high elongation suggest a ductile behavior, Fig. 5.4. A neck in the circular area is observed after deformation, Fig.5.5-1c, which suggests the sliding of nanotubes in a way similar to relaxed polymer chains. In fact, the nanotubes are aligned with nanospheres, forming pearl-like chain domains which shape the fibrils, Fig.5.5-1d. We thus distinguish the contribution of nanospheres to this particular deformation. SEM micrographs taken on tested samples, Fig. 5.5-1c,d., corroborated with AFM topographic view, Fig.5.3-c,d describe this sequence. Images taken both alongside of fiber and perpendicular to it show that the SW-RG503H-2 fiber topography is characterized by the presence of many nanotubes on the fiber's surface. By comparing the topography of this fiber with that of SW-RG503H-1, Fig. 5.5-1a,b, we illustrate different disposition of nanotubes, which could be at the origin of this particular mechanical behavior. This particularity is one of the facets of our

formulation and could be attributed to the relaxation of fibrils. In the case of SW-RG503H-2 sample, the fibrils are looser in comparison to the SW-RG503H-1 fiber. After fracture, the surface of the SWRGH-1 fiber is rough suggesting a very strong interface, Fig. 5.5.-1a. The fibrils organized at submicroscopic level include nanotubes and nanospheres which remain together even after the fracture. A similar behaviour is revealed for the SW-RG502-1 sample, Fig. 5.5-2a, where the spherical shape is distinguished on the fractured surface after the nanotubes slide. The sample SW-RG502-2, has the highest nanotube' content which seems strengthen its structure. In these fibers, SWNTs are interconnected with PLGA nanospheres packing, thus forming a unified CNT network which sustain the fiber. The mechanical response could be also associated to the roughness of fibers. Using AFM measurements we determine the fiber roughness; for 500x500 nm area, we distinguish a roughness of 48 nm, for the SW-RG503H-2 sample, which is the highest value, while in the case of SW-RG503H-1 it is of 12, 3 nm, Table 5.1. This difference strengthens our hypothesis that, in the case of SW-RG503H-2 sample, the nanotubes and nanospheres are not closely packed, with the consequences on the nanotubes mobility, further reflected in mechanical behavior of macroscopic fiber. A good attachment between nanotubes and spheres is also suggested by the surface profile after testing, Fig. 5.5-2c. The assemblies of nanotubes-nanospheres are deformed together and pearl-like structures are observed on surface.

Results of the measurements of dynamical properties, which define a structure-property relationship, provide a new proof of the structural organization of nanotubes into fiber. We performed dynamical mechanical analysis (DMA), in tensile mode, at the

constant frequency. The results are presented in Table 5.3, and evidence significant increases in the elastic modulus due to the presence of aligned SWNTs. The variation of the storage modulus (G') is an indicator of the elastic behaviour while the loss modulus, G'', measures the viscous behaviour of the fibers. An example is presented in Figure 5.6-a. The maximum values of G'' correspond to the glass transition (T_g) of new fibers whose values are presented in Table 5.3. Corresponding to the literature, the T_g values measured by DMA are higher with 5-7 °C than the values measured by DSC. Taking into account this note, we observe that the sample SW-RG503H-2 has a real Tg very close to the body temperature, which is also the temperature at which we performed cell culture. It is well known that above this temperature the material displays increased visco-elastic behaviour. The fibers SW-RG503H-1 and SW-RG502-2 have the highest values of Tg. In the case of the latter this could be related to the higher content in CNTs, 30%, while in the first case this is due to the contribution of PVA, as indicated by the thermal profile presented in Fig. 5.2. In fact, the preparation of the SWGR502-2 sample involved a more concentrated PLGA suspension. This implies a higher density of nanoparticles inserting more bundled carbon nanotubes which is detrimental for the interface strength at the molecular level. Also, an increased number of nanoparticles signify more adsorbed PVA on nanospheres with the tendency to form agglomerates thus increasing the surface roughness, measured for 10x10 μm and 5x5 μm areas, Table 5.1. Hence, we can explain why an 62,5% increase of CNT content, in SW-RG502-2 sample, does not enhance the elastic modulus, tensile stress, nor strain, in comparison with the other three samples. We suppose that this effect is caused by the development

of a weak interface between nanotubes and spheres which is not helpful for the transfer of the load to nanotubes. Although aligned, the nanotubes are closer each others forming the bundles. The cross section images of fibers before, Fig.5.1.-1d, and after mechanical testing, Fig.5.5-2d, depict the state of the nanotubes. We observe the bundled nanotubes which pull out after the test with the formation of cavities or isolated spheres. In these conditions the content of nanotubes, even though, does not achieve a maximum performance.

The higher value of T_g corresponding to SW-RG503H-1 sample is justified by the presence of a strong interface between nanotubes and PVA. It has been demonstrated that fibers containing only SWNTs coagulated with PVA polymer display a Tg at 76°C, [15] that is close to the Tg of PVA, about 80°C. These values originate from the crystallinity of PVA. Our fibers are characterized by the presence of PLGA nanoparticles interlaced with PVA chains and nanotubes. This composition and particular arrangement of the fibrous structures changes their thermal behaviour. The PLGA copolymers we use have a Tg of 40 °C and 38 °C, respectively. An amorphous copolymer, the PLGA influences the behaviour of the new fibers, by decreasing the glass transition temperature related to the presence of PVA and introduces a new glass transition temperature. The lowest value is that of the SWRG503H-2 fiber. The variation of the loss factor, tan δ, as a function of temperature, provides information that support the structure-properties relationship, Fig.5.6-b,c. The measurement of tan δ, as a function of temperature shows the increase of its values at a temperature of 35-45 °C.

For SW-RG503H-1 and SW-RG503H-2 this variation is less visible than that observed for samples SW-RG502-1 and SW-RG502-2, suggesting more close-packing between the nanotubes and nanospheres. Generally, the SW-RG503H samples show more closed nanosphere-nanotube packing while more relaxation could be assigned to nanotubes in SW-RG502 samples. This difference, although not very significant, possibly will be explained in terms of interface. In fact, the copolymer RG503H is an uncapped copolymer with carboxylic end-chains. This end-chain architecture enables more interactions with nanotubes thus reinforcing the interface. At the same time, the availability of PLGA nanoparticles for PVA adsorption is reduced and the content in polymer component will not be very different.

The mechanical behaviour, as presented herein, is not restrictive for our fibers in their interaction with cells. The content of PVA and PLGA endows fibers with high flexibility in aqueous media. This flexible behavior has been evidenced even during the handling of thread in the spinning process and the subsequent steps. This effect is also increased by the role of water as a plasticizer for PLGA, which is a great advantage during the exposure of fibers to cells. Moreover, the low miscibility of PLGA with water palliates the tendency of PVA to swell in water thus providing fiber stability during the culture. A correlation between the thermal behaviour and morphology enable us to predict the high performance of these fibers as a support for the fibroblasts growth.

5.4 *in vitro* BIOCOMPATIBILITY

When designing fibers as supports for the cell growth, particular consideration must be given to the structural and morphological characteristics, as it is known that substrate ordering and structure have a direct impact on cell accommodation on the surface [16,17]. Once introduced in the human body, in their nanoscopic size, CNTs, are able to move unhindered throughout the entire body with possible negative consequences. Our achievement in nanotubes integration in a macroscopic form, as reflected by fiber' nanoscale characteristics, is a guarantee for the fiber' efficiency as a support for fibroblast growth, with great benefits from the biocompatibility point of view.

Cell adhesion and migration involve the action of the contact guidance components on topographical characteristics of fibers. Human skin fibroblasts were cultured for three days onto the fibers. Using optical microscope we observed the adhesion and migration of cells daily. As presented in Fig. 5.7-1, the presence of fibers in the culture media encourages the alignment of cells and increases their density near the fibers thus suggesting the latter's role in inducing cell alignment. The fibers are not transparent therefore we can not observe the cells on the fibers. One of the phenotypic features of fibroblasts is their elongated cell shape. Physical profile of substratum induced the alignment or directional growth as depicted by micrographs, Fig. 5.7-1. The images suggest a change in cell morphology in relation with the fiber support; for certain samples cells develop many filopodia typical for cell sensing surrounding, Fig. 5.7-2a. For other samples cells remained spherical in shape accompanied by the formation of

small lamellipodia, Fig. 5.7-2b. Micrographs taken with FM depict the growth of nuclei

after three days of culture and evidence the concentration of cells when adjacent to the

fibers, Fig. 5.8-1. Most cells spread significantly on the SW-RG503H-2 fibers, Fig. 5.8-

1b. The MTT test is a very sensitive method for the assessing biocompatibility and the

results we obtained prove the ability of fibers to maintain and even slightly improve the

viability of cells without a sign of cytotoxicity, Fig. 5.8-3. The performance of the fibers

as alignment inducers for cells and their impact on cell morphogenesis is revealed, Fig.

5.7-1. Cell migration plays an essential role in colonisation of fiber biomaterial. The

examination of fiber migration shows the role of SWNT fibers as a cue in the motricity

of cells. We evidence that topography influences and directs cell migration, more

particularly the discontinuities created by the interference of longitudinal roughness with

the lateral roughness, Fig. 5.9-d. Cells migrate from the original location to other

locations within the first 2 days. In fact, using directionally aligned CNT fibers, we

enable to maintain one direction of migration; the fibers are 3D-like support with

unidirectional alignment. Two types of orientations are observed: i) at macroscopic level

which regards the fiber shape in their environment, Fig.5.9-c; ii) the alignment of carbon

nanotubes at micro and sub-micro level which enhance the contact-guide with individual

cell, Fig.5.9-a,b. After two days of culture the area not seeded with cells was covered by

them due to their migration, Fig. 5.8-2(3). The cells have an elongated shape and

continue to migrate in the opposite direction of the sample. A great incitation for

migration is conferred by the SW-RG503H-1 fiber support and we attribute this success

to the fiber rigidity. In fact, when in the direct contact with cells, the flexibility of

substrate is beneficial for their growth [17]. At the same time, a fibrous substrate with diameter of 30-50 μm has to stay stable in an aqueous environment where the stiffness of fiber is a requirement. Thus, the fiber stiffness could explain the successful migration. We note that, in an aqueous environment, at 37°C, the elastic modulus, could be even lower, knowing the role of water as a plasticizer for PLGA copolymer. Through their shape, these macroscopic fibers are 3D arrangements of fibrillar elements structured in a macroscopic cylindrical form. SWNTs interconnected with spherical shapes form fibrillar units with webbing interconnections and a unidirectional orientation. The use of this fibrilar network in an oriented shape provides us with the ability to create 3-D structures that supply alignment cues to cells. In these structures, aligned nanotubes are an asset.

The roughness of fibers plays a significant role in the fiber contact with cells. We can observe a variability of the forms alongside and across the fiber, Fig. 5.9-a,b. The interference of these geometric details creates a periodic textured surface, Fig. 5.9-c,d, with beneficial effect on cell adhesion. By combining structural and morphological features we provide an advantageous way to enhance interactions with cells. The creation of a specific topology and spatial characteristics endows these fibrillar structures with the capacity to establish favourable interactions, when in contact with fibroblasts. The latter are the main components of the ECM, characterised by its stiffness.

5.5 CONCLUSION

By formulating these fibers we feature four main particularities in the relation support-cell with a corresponding factor-effect relationship: i) fiber shape; ii) scale effect concretised in nanosized surface texture and micro-fibrillar structure; ii) bulk characteristics consisting of compositional involvement of SWNTs and PLGA nanoparticles; iv) multilevel architecture directly related to the hybrid integration of nanotubes through the colloidal mixture. The hybrid approach used to integrate the nanotubes at nanometric level enhances the translation of their intrinsic characteristics with a beneficial effect on their biocompatibility.

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ACKNOWLEDGEMENTS

LEGEND OF FIGURES

Figure 5.1   **SWNT Fibers' Structure and Surface Topography.   1-a,b,c,d:** SEM micrograph depicting cylindrical shape of SWRGH-1, SWRGH-2, SWRG-1 and SWRG-2 fibers and their homogenous structure as viewed by the cross section. **2-a,b,c,d:** Taping mode AFM topography measured along the SWRGH-1, SWRGH-2, SWRG-1 and SWRG-2 fibers, at a 500x500 scan areas; the arrows indicate the nanotubes bundles .

Figure 5.2   **Thermal Stability of SWNT Fibers as determined by TGA measurements.** The loss of weight of SWRGH-1, SWRGH-2, SWRG-1 and SWRG-2 fibers was measured as a function of temperature.

Figure 5.3   **Morphological Characteristics of SWNT Fibers.** Topographic AFM images, in taping mode, taken along and across the SWRGH-1, SWRGH-2 fibers reveal the topographic effects created by the presence of PLGA nanoparticles. Phase images alongside of SW-RG503H-1 and SW-RG503H-2 fibers (e,f) demonstrate the different distribution of nanotubes, as indicated by the arrows.

Figure 5.4   **Mechanical Behaviour of SW-RG503H-1 and SW-RG503H-2 Fibers.** Stress-Strain curve reveals the particularities of each type of fiber.

Figure 5.5   **Structural Changes of SWNT Fibers Induced by Mechanical Testing.**
SEM micrographs depict structural changes of samples after submitted to
the mechanical test: **1.** SW-RG503H-1 and SW-RG503H-2 at two different
magnifications after testing; **2.** SW-RG502-1 and SW-RG502-2 at two
different magnifications after testing.

Figure 5.6   **Dynamic   Mechanical   Behaviour   as   Determined   by   DMA
Measurements. a.** Variation of G' and G'' as a function of temperature
measured for SW-RG503H-1 sample; **b.** Evolution of loss factor, Tan δ, as
a function of temperature for,   SW-RG503H-1   and   SW-RG503H-2
samples; **c.** Evolution of Tan δ, for SW-RG502-1 and SW-RG502-2
samples.

Figure 5.7   **Fibroblats' Growth and Cell Attachment. 1.** Optical images taken for
SW-RG503H-1, SW-RG503H-2, SW-RG502-1 and SW-RG502-2 fibers,
after 3 day culture period, in the presence of skin fibroblasts. Original
magnification: x200. **2.** Two different evolutions of fibroblasts, imaged
with SEM after 3 day culture period, being in direct contact with SWNT
fibers; the arrows indicate the site of contact of cell-nanotubes..

Figure 5.8   **Proliferation of Fibroblasts and Cell Migration. 1.** FM images taken for Hoechst stained SWRGH-1, SWRGH-2, SWRG-1 and SWRG-2 fibers after 3 day culture period in the presence of skin fibroblasts. Original magnification: x100.   **2.** Cell Migration after 2-day of culture in the presence of SWRGH-1 fibers. Original magnification: x200.

Figure 5.9   **Topography and Texture of SWNT Fibers. a,b.** AFM 3-D topographic view of SW-RG503H-2 and SW-RG502-2 fibers' surface; **c.** SEM micrograph depicting the texture of SW-RG502-1 fiber; **d.** Scheme illustrating the interference of geometric details to form variable textures as function of the size of nanoparticles and their arrangement.

LEGEND OF TABLES

Table 5.1.

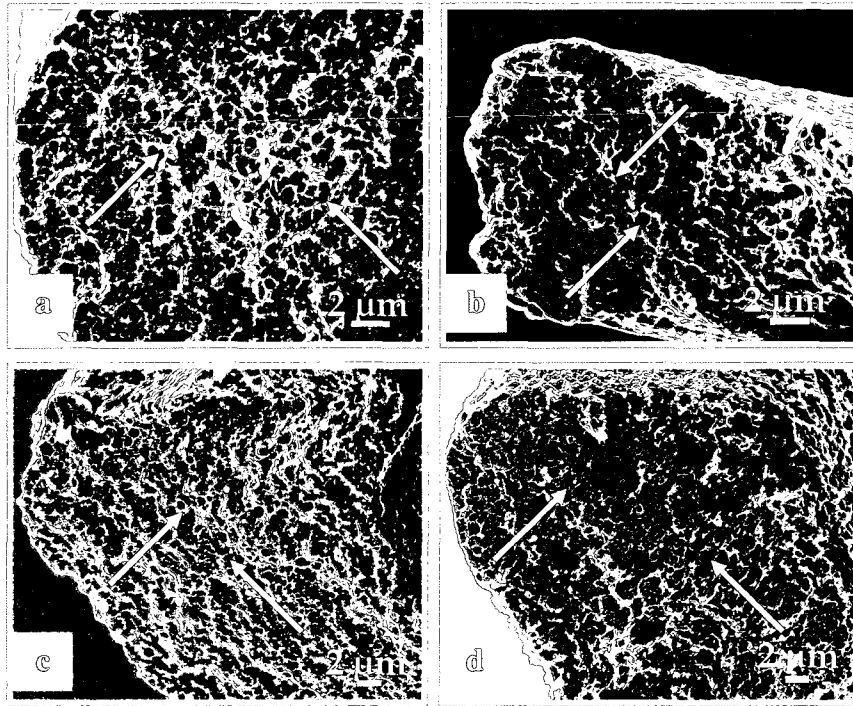Fiber roughness as determined by AFM measurements along and across SWNT Fibers.

Table 5.2.

Mechanical Characteristics of SWNT Fibers

Table 5.3.

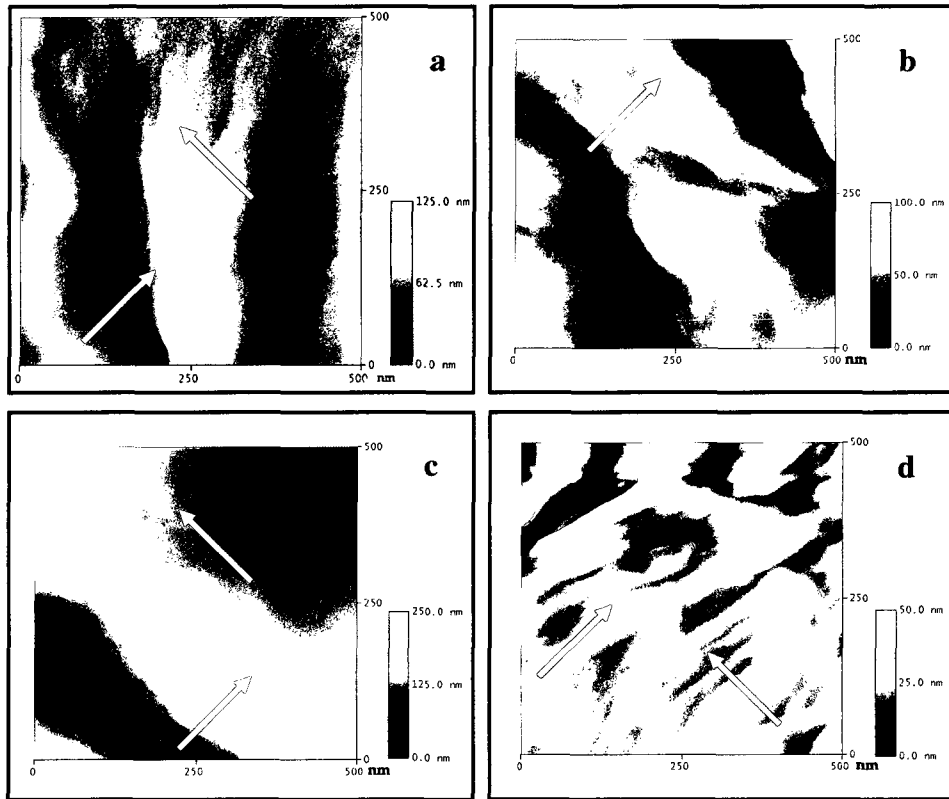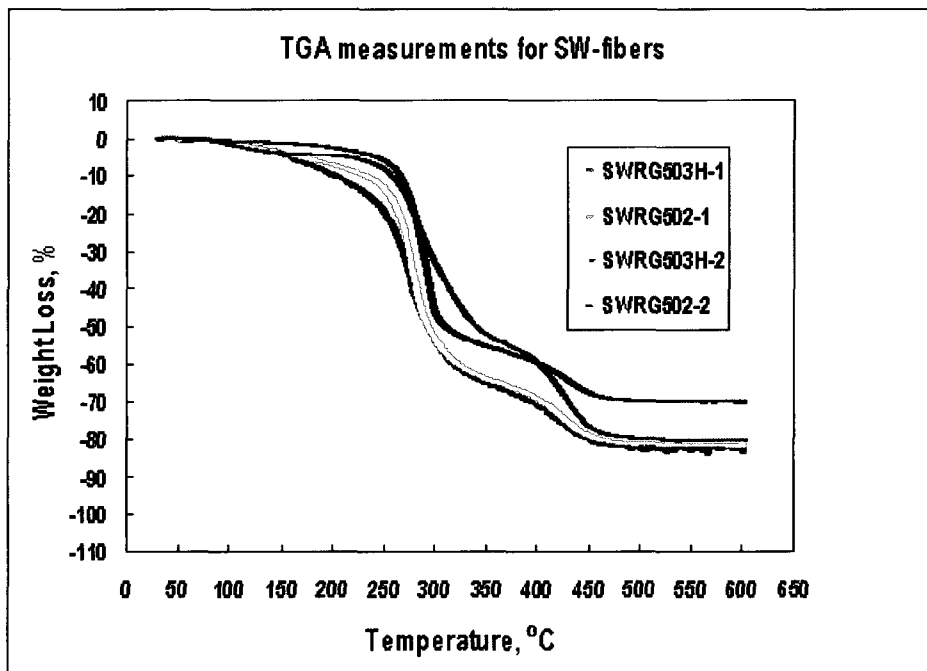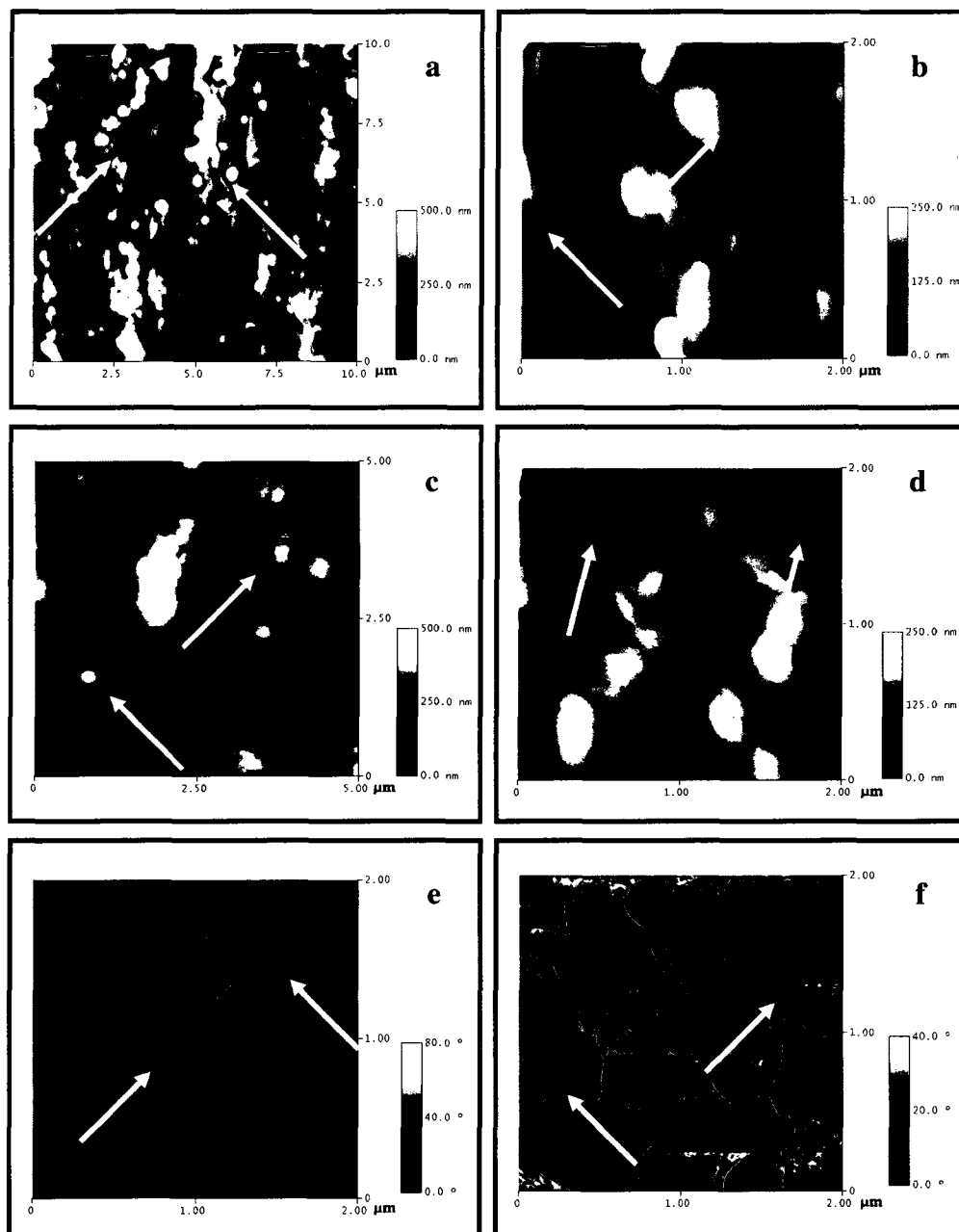Dynamic Mechanical Characteristics for SWNT Fibers

1

2

Figure 5.1

Figure 5.2

Figure 5.3
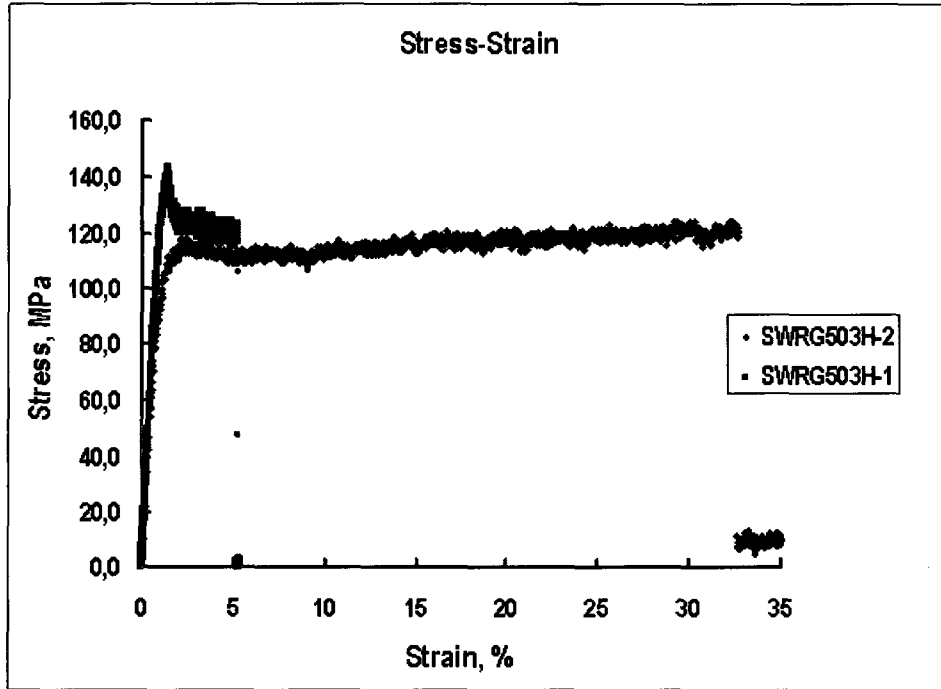
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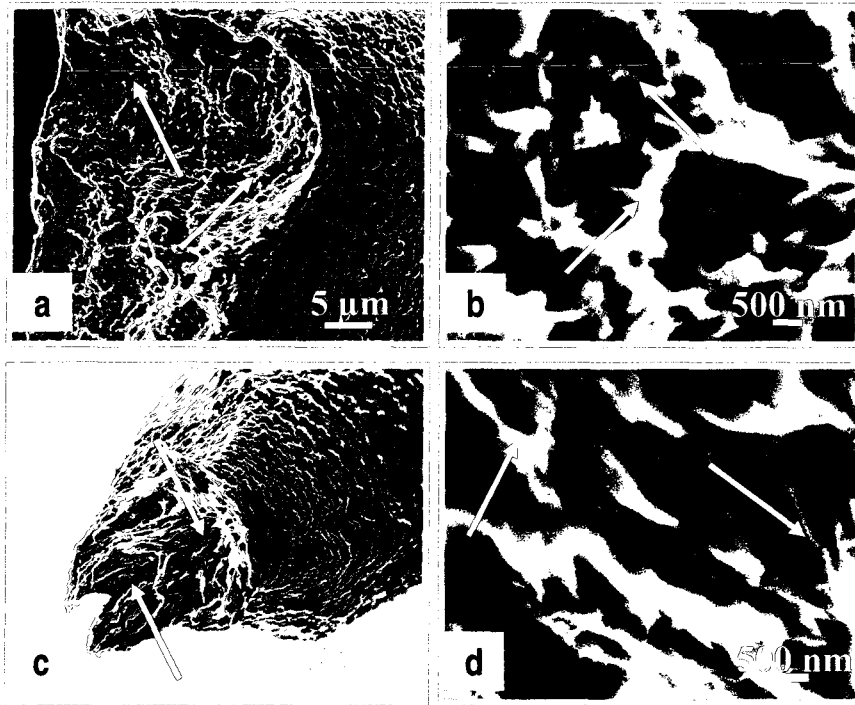
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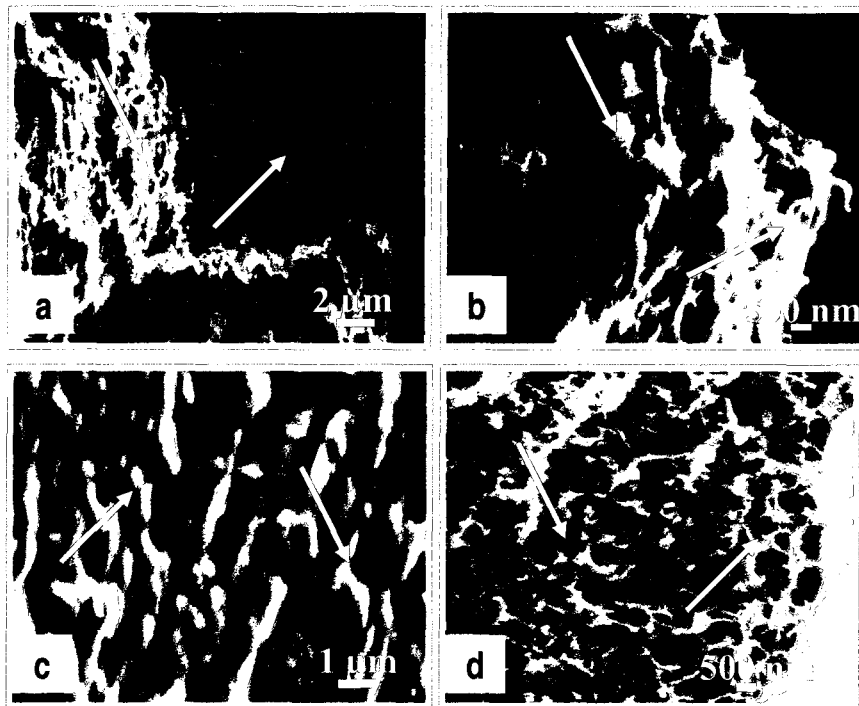
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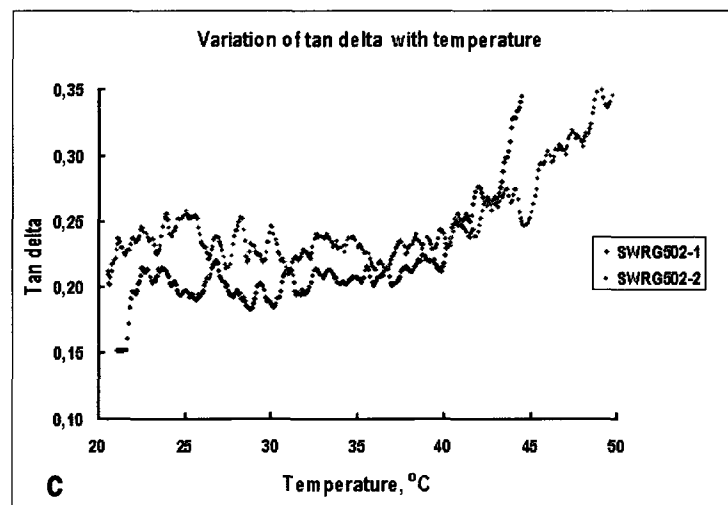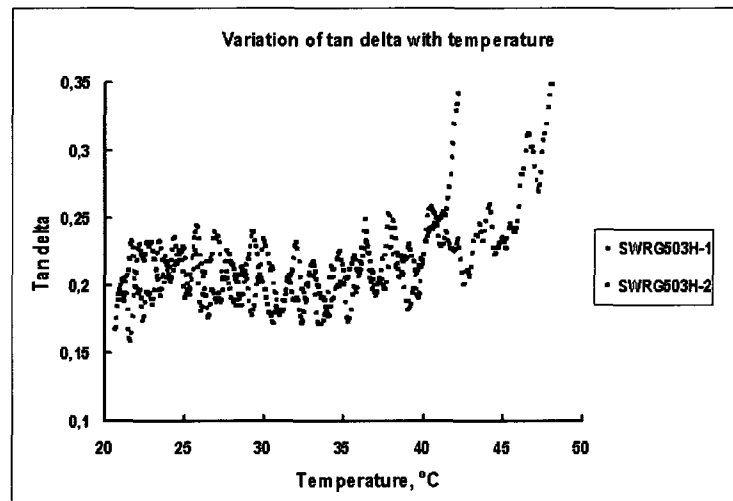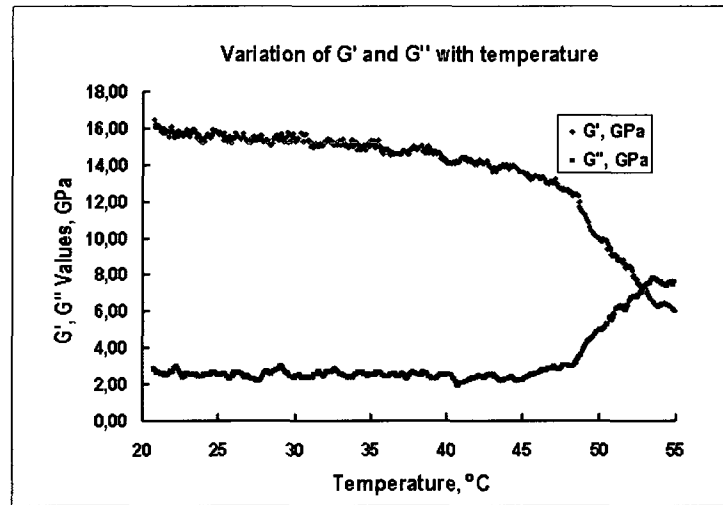
Figure 5.6

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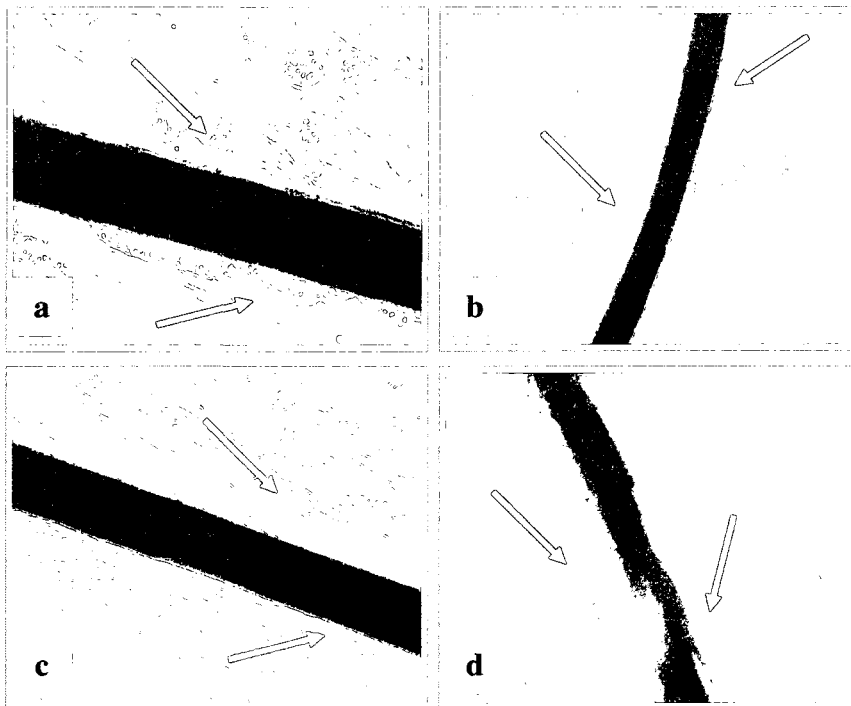
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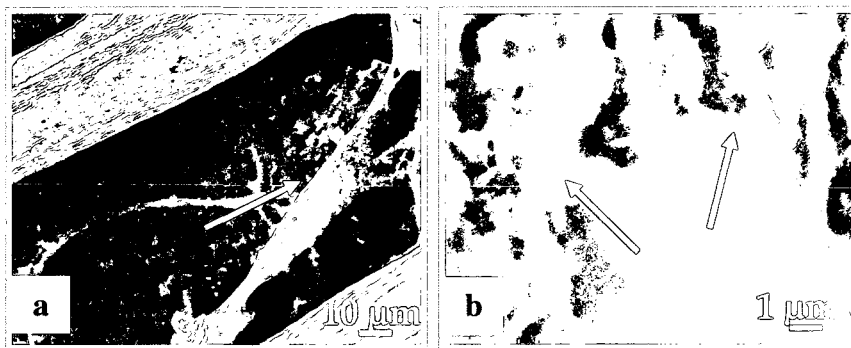
Figure 5.7

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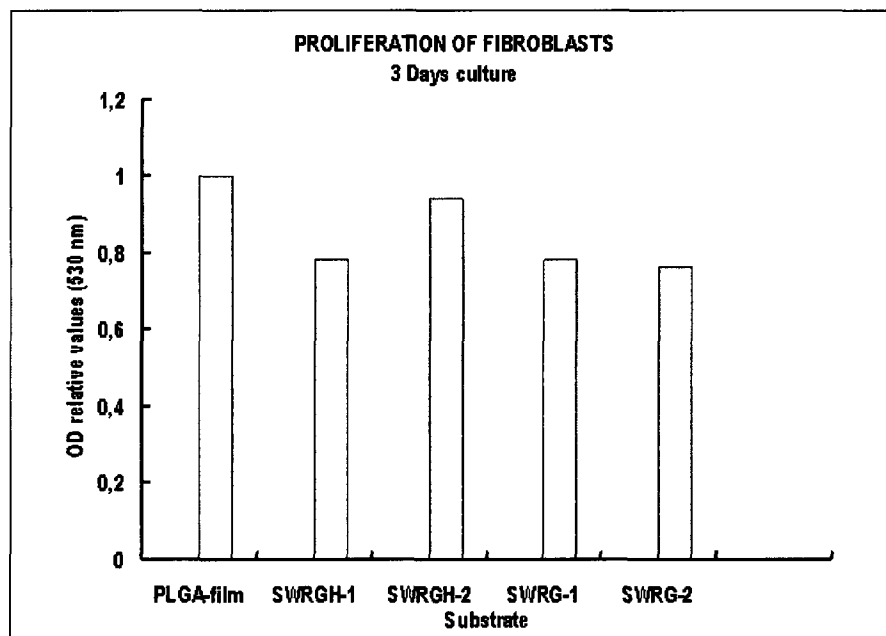
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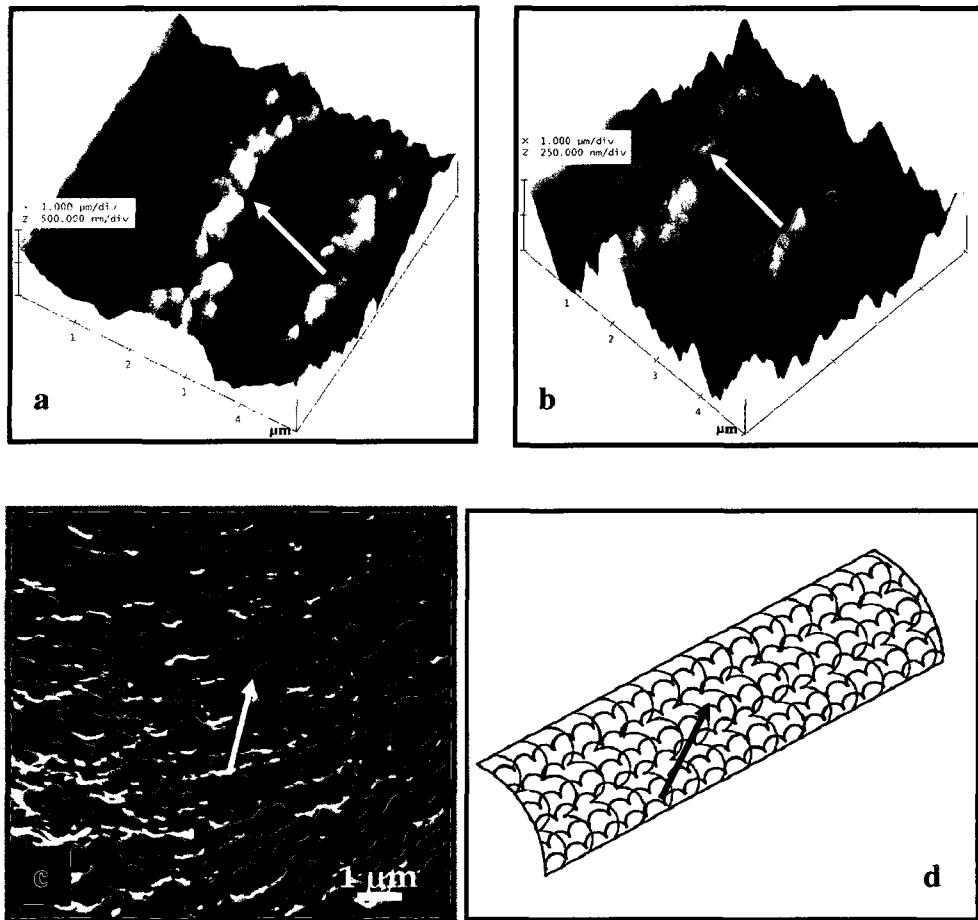
Figure 5.9

TABLES

Table 5.1.

Fiber Sample	Roughness (RMS) 0°			Roughness (RMS) 90°	
	10x10 μm area nm	5x5 μm area nm	0,5x0,5 μm area nm	5x5 μm area nm	0,5x0,5 μm area nm
SW-RG503H-1	83,2	94,7	12,3	73,4	0,3
SW-RG503H-2	104,8	159,4	48,8	67,1	3,0
SW-RG502-1	114, 9	74,7	25,3	54,4	7,9
SW-RG502-2	124,8	108.6	8,2	30,0	6,7

Table 5.2.

Fiber Sample	Stress (σ) MPa	Elastic modulus (E) GPa	Strain (ε) %	CNTs Content (%)
SW-RG503H-1	150	18.0	2.5	18,5
SW-RG503H-2	140	13.0	30	19,6
SW-RG502-1	140	11.5	2.0	18,5
SW-RG502-2	130	11.0	1.9	30,0

Table 5. 3:

Fibers Sample	G' GPa		G'' GPa		Tg, °C determined by DMA
	Max. value	at 37°C	Max. value	at 37°C	
SW-RG503H-1	16	14,8	7,43	2,45	53
SW-RG503H-2	9,3	8,9	4,5	1,8	45
SW-RG502-1	13,2	11,8	6,5	2,5	48
SW-RG502-2	12	10	3,4	1,0	56

CHAPTER 6

NANOSCALE SURFACE OF CARBON NANOTUBE FIBERS

FOR MEDICAL APPLICATIONS:

STRUCTURE AND CHEMISTRY AS REVEALED BY TOF-SIMS ANALYSIS

TITLE PAGE

Nanoscale Surface of Carbon Nanotube Fibers for Medical Applications:

Structure and Chemistry Revealed by TOF-SIMS Analysis

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6.1. ABSTRACT

Surface structure and related chemistry understanding is a vital element in the design of high biocompatible materials since adsorption and adhesion of biological components are involved. These features are even more important in the case of nanostructured materials such as carbon nanotubes (CNTs) fibers. In our preliminary work we synthesised CNTs based fibers for medical applications. This new hybrid system combines polyvinyl alcohol (PVA) with CNTs and polylactic-co-glycolic acid (PLGA), a biodegradable copolymer. The surface properties of this material are investigated in order to guarantee a biocompatible response. Time of Flight Secondary Ion Mass Spectrometry (TOF-SIMS) was found to be an ideal tool for fiber characterisation owing to its capacity to provide chemical specificity combined with detection limits beyond the reach of techniques previously used. Complementary morphological information is provided by Atomic Force Microscopy (AFM). The corroboration of both data enables us to define the chemistry and structure of this new formulation.

6.2. INTRODUCTION

A growing interest toward the field of hybrid materials is currently observed in the biomedical world. The hybrid approach provides great fabrication versatility and encourages the creation of new materials with unique properties, depending on the nature of the incorporated functional segments. CNTs are emerging materials which capture a great interest in the biomaterial field [1]. It is noteworthy that the effectiveness of CNT in hybrid materials strongly depends on the ability to disperse the nanotubes homogenously through the matrix while maintaining their integrity and ensure bonding between components [2-4].

We engineer CNTs fibers applying Particle Coagulation Spinning (PCS) method which forms macroscopic fibers with a very good alignment of nanotubes [2]. Using CNT dispersion based on a non-covalent stabilisation mechanism, in aqueous solution, we ensure the preservation of both the nanotube structure and its properties. Incorporation of PLGA in CNTs dispersion strengthens the network effect and further increases the biocompatible response [5]. When constructing fibers for biomedical application, surface chemistry and morphological homogeneity are of particular significance for such complex materials since they directly influence biological interactions [1,6]. Indeed, the structural features and the chemical composition at the fiber surface are crucial for understanding specific functional properties which have a great impact on tissue regeneration [1].

TOF-SIMS has great surface specificity and high detection limit and shows differences in spectra for different states of the various components of the fiber biomaterials [6]. The

present study is concerned with the nanoscale surface characteristics of CNTs based fibers. The properties of the studied hybrid structure, in terms of biocompatibility, strongly depend on the components' chemistry such as CNT, PLGA and PVA, as well as on the fibers' structure. A variety of other reports refer to the characterisation of CNTs based materials using various techniques [2-4]. In this frame of investigation, surface chemistry analysis has not yet been well studied. In order to explore the chemistry of this hybrid system we examined the surface using TOF-SIMS. Static TOF-SIMS is a surface analytical technique that uses a primary ion beam to bombard a sample and sputter characteristic mass fragments from the sample's surface. In this study, we applied TOF-SIMS to measure the relative presence of chemical species in the fibers top monolayer. TOF-SIMS imaging is used to generate chemically well-defined images of the studied fibers. We were specifically interested in investigating whether the three components of the hybrid system are present on the surface and dispersed homogenously.

6.3 EXPERIMENTAL

Carbon Nanotube Fibers

The CNT based fibers constituted in a hybrid material system are obtained by blending single wall carbon nanotubes (SWNT) dispersion, PLGA and PVA in aqueous solution. The SWNT suspension is prepared using sodium dodecyl sulphate (SDS). This surfactant, with chemical formula $C_{12}H_{25}O_4SNa$, absorbs at the surface of SWNT

bundles. The PCS process ensures the interconnection of polymer chains and CNTs into fibers with variable length and diameter ranging from 50 to 70 μm [2]. In our new approach, PLGA copolymers, with two different molecular weights, were added in order to obtain a new biocompatible biomaterial, with two different formulations [5]. Three types of samples were analysed and are identified as the following: 1) SWC (control) containing CNTs and PVA; 2) SWA containing CNT, PVA and PLGA (M_w = 28 kDa); 3) SWB containing CNT, PVA and PLGA (M_w=12 kDa). The control fibers were obtained following the current method [2]. Both SWA and SWB new types of fibers were prepared using two different molecular weight copolymers as presented in the formulation process [5]. The copolymers were produced by Bohentingher Inghelheim and the choice of molecular weight was based on specific considerations. The Hipco SWNTs used in the processing were purchased from Carbon Nanotechnologies Inc., in the form of purified powder. The analysed samples were presented as yarn with a diameter ranging in of 50-60 μm and a length of 70 mm.

Atomic Force Microscopy: Observation of surface morphology was carried out with an AFM, Digital Multimode Scanning Probe Microscope. The measurements were performed in contact mode using a tip with a radius of 40 nm, for a selected area of 2 x $2\mu m^2$, 5 x 5 μm^2 and 10 x 10 μm^2, respectively, in order to obtain submicrometer morphological features of the general section.

Time-of-flight secondary ion mass spectrometry: The work was carried out using TOF-SIMS IV apparatus (ION-TOF GmbH, Münster, Germany) in bunch mode. Samples were analyzed at a pressure of approximately $1.5 \times 10\text{-}9$ Torr and bombarded with $^{69}Ga^+$ primary ions at energy of 15 keV. SIMS operates with a 1.25 pA beam current rastered over a frame area 19 x 19 μm^2 along the length of fibers with a spatial resolution of 100 nm. The gun was operated with a 27.5 ns pulse width, for an ion dose of 7.8 $x10^8$ ions cm^{-2}, lower than the threshold level of 1 $x10^{13}$ ions cm^{-2} for static conditions. Effective charge compensation was obtained by using the electron flood gun. Secondary ions were detected with a Reflectron time-of-flight analyzer, a multichannel plate (MCPs), and a time-to-digital converter (TDC). Measurements were performed with an acquisition time of 100 s, at a TDC time resolution of 200 ps. A spectral analysis using the provided software was performed to identify the characteristic ions associated with peaks in the mass spectrum. Peak intensities were measured as the Poisson-corrected area of the peak, normalized by the total counts of the spectrum. Characteristic ion masses were used to reconstruct an image of the analyzed fiber area.

6.4. RESULTS AND DISCUSSION

This study is a new application of TOF-SIMS to CNTs based fibers analysis. Our method relies on the technique's ability to identify molecular fragments of organic molecules and inorganic ions. The positive and negative spectra of fibers were measured and compared. Characteristic ions are related to main components of fibers: CNT, PLGA and PVA as well as SDS. The ion's individual contribution to the fiber

composition is considered in term of intensity, as presented in examples from Table 6.1 and Table 6.2. TOF-SIMS detects the differences in spectra for different states of fibers. The specificity of each fragment, in both positive and negative mode, reveals the participation of every chemical species to the composition of the hybrid system as indicated below. TOF-SIMS is able to diagnose a specific signature involving copolymer molecules whose contribution is visible in the case of SWA and SWB fibers. Their detection demonstrated a successful interconnection and the coexistence of all components at the surface, resulting in the formation of a strong, well organized network.

The specific peaks in both positive and negative mode confirm the presence of PLGA at fiber surface. The negative ions are found in different ratios and we attributed some specific ions to the copolymer with higher molecular weight (A: m/z = 73, 75, 89) as resulted from PLGA fragmentation, Table 6.2. Another indication for the presence of copolymer is related to the positive ions $C_3H_5O_2^+$ and $C_6H_{11}O_4^+$ corresponding to m/z = 73 and 147 respectively, and identified as PLGA fragments. We are also able to identify the presence of SDS surfactant through sulphur related positive and negative ions, CHS^+, $C_3H_7S^+$ and $CHSO^-$. In fact the concentration of SDS is the same within all SWNT dispersions and its variation in the fibers is due to introduction of copolymer which changes the ratio between components. Additionally, the presence on the surface of SDS molecules, playing a critical role in the phase behaviour [2], demonstrates our capacity to formulate the macroscopic fibers of SWNT with a hybrid composition.

Indeed, the TOF-SIMS technique, with its sampling depth of 1-2 nm, provided SDS molecular ions in both positive and negative mode, as presented in Table 6.1 and Table 6.2

TOF-SIMS imaging was used in order to demonstrate the ability of this new approach to homogenously distribute each component in the hybrid system, with respect to the external surface.

Images of one relevant molecular ion were presented in Fig. 6.1 to illustrate the uniform contribution of the PVA polymer chains to the fibers. The most important presence is reliable to SWC sample and, in agreement with the formulation; it diminishes with the introduction of copolymer in SWA and SWB fibers. In the case of inorganic ions, we focused on Fe^+, considered as a trace of the catalyst used in the synthesis of SWNT. The variation of its concentration is linked to the formulation of SWA and SWB respectively, as presented in Fig. 6.2. The appearance of these ions is more visible in the control sample than in the new fibers. This information indicates a consistent dispersion of carbon nanotubes in fibers and the material homogeneity as well as the changes induced by copolymer intercalation. The chemical configuration, as suggested by TOF-SIMS imaging, is corroborated by the morphological description realised by AFM analysis which shows well organised, oriented nanoscale architecture. This is complementary information supporting the homogenously organised fiber surface. The examples presented in Fig. 6.3, correspond to $2 \times 2\mu m^2$ area measurements and they show the evolution of fiber microstructure with a new formulation; the finest structure

was obtained in the case of SWA fibers. The SWA surface clearly shows a more fibrillar nanostructure in the range of 10-20 nm while on SWB surface the structural units are larger and less homogenous. The both SWA and SWB structures are different from SWC surface which rather consists in linear segments than fibrillar units. These AFM images sustain the contribution of copolymer to nanoscale structure of thread, thus influencing its morphological features.

6.5. CONCLUSION

To summarise, the performed surface characterization demonstrated specific surface chemistry and structure for the CNT based fibers we produced. Qualitative interpretation of the spectra reported in this work allows us to define a characteristic fragmentation pattern for the new fibers when compared to the control. It appears that copolymer fragments have a significant contribution to the spectrum. TOF-SIMS imaging proves not only the homogenous distribution of organic and inorganic ions but also registers the variation of each component as function of formulation. AFM data revealing surface morphology and texture was used as a complementary tool to describe fiber microstructure and significant changes induced by using a third component, the PLGA copolymer. Our results demonstrate that the identification of various chemical species at the surface is a useful way to illustrate the realisation of nanostructured fibers in a new hybrid system, with the contribution of three components: SWNT, PLGA copolymer and PVA matrix. Knowing the chemistry and morphology of the surface enables close

monitoring of interactions with the body, and consequently the impact on biocompatibility.

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LEGEND OF FIGURES

Figure 6.1   Variation of $[C_2H_3O_2]^-$ Molecular Ion on Fibers'Surface as a Function of

the Formulation

Figure 6.2   Variation of Fe^+ Ions on Fibers'Surface as a Function of the Formulation

Figure 6.3   AFM Topographic view in Contact Mode for Different Fibers

LEGEND OF TABLES

Table 6.1   Positive Ions as determined by TOF-SIMS

Table 6.2   Negative Ions as determined by TOF-SIMS

FIGURES:
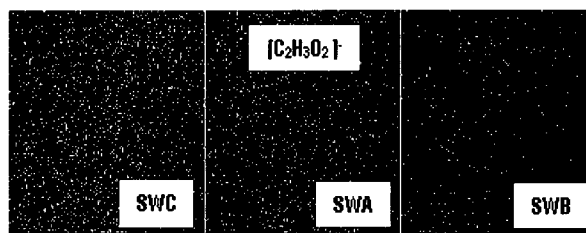
Figure 6.1
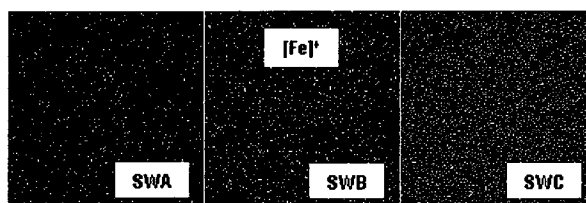
Figure 6.2
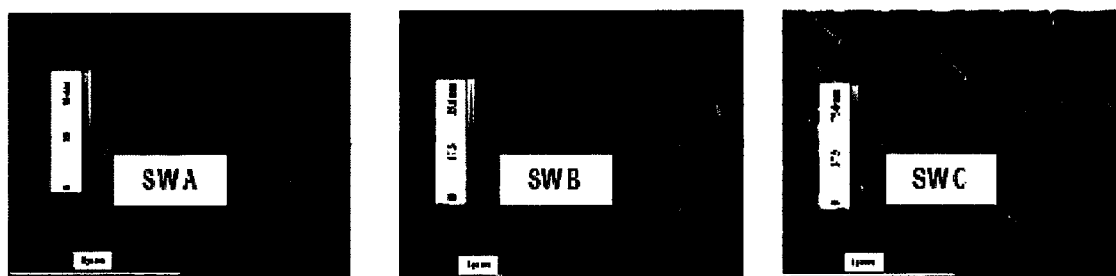
Figure 6.3

TABLES

Table 6.1

Ion	Ion Mass	Molecule Origin	Intensity, counts x 10^3		
			SWA	SWB	SWC
$C_2H_2^+$	26	CNT, PVA	0.6	0.6	0.5
$C_2H_3^+$	27	CNT, PVA	2. 3	3.3	1.9
$C_2H_3O^+$	43	PVA	8 .0	7 .8	9.5
CHS^+	45	SDS	2.3	1.6	1.9
$C_4H_7^+$	55	PVA	0.2	0.8	0.3
Fe^+	56	CNT	0.15	0.13	0.4
$C_3H_5O_2^+$	73	PLGA	10.9	10.6	0.0
$C_3H_7S^+$	75	SDS	0.4	0.7	1.0

Table 6.2

Ion	Ion Mass	Molecule Origin	Intensity, counts x 10^3		
			SWA	SWB	SWC
C_1^-	12	CNT, PVA	3.9	2.8	7.5
O^-	16	CNT, PVA	29.0	16.0	43.0
C_2^-	24	CNT, PLGA	3.7	2.2	10.3
C_2H^-	26	CNT, PLGA	11.6	5.6	26.0
$C_2H_3O_2^-$	59	PVA	1.1	0.2	1.6
$CHSO^-$	61	SDS	0.6	0.2	0.9
$C_2HO_3^-$	73	PLGA	0.2	0.0	0.0
$C_2H_3O_3^-$	75	PLGA	1.1	0.3	0.0
$C_3H_5O_3^-$	89	PLGA	0.4	0.0	0.0

CHAPTER 7

GENERAL DISCUSSION

The general aim of this research work was to create a new biomaterial containing carbon nanotubes with biodegradable behaviour and a biocompatible response in relation to the neural applications. The work has been focused on the exploiting the wet spinning process as simple method for the integration of CNTs in macroscopic fibers for the design of a new approach personalized for the fabrication of biomaterials containing CNTs. Four specific objectives were formulated through the strategy for the new formulation.

Our initial purpose was to explore the new structures of carbone nanotube and their unique characteristics with potential for biomedical applications. The capacity of these structures to help the growth of neural cells was identified with the aim to establish the principle of a new formulation for the neural biomaterial. The review of CNTs emphasizes the characteristics of CNTs as biomaterials while focusing on their prospective applications in medicine, in biology and biomedical engineering. The intrinsic properties of carbon nanotubes are presented in order to introduce the most interesting aspects for the biomaterial field. A clear understanding of structure-properties relationship is considered as well. This description regards the structural particularities,

the physical, the chemical and the electronic characteristics along with the mechanical behaviour. The chemistry of CNTs focuses on the chemical inertness of nanotubes and present multiple methods used for their chemical modification. The particularities of both the covalent and non-covalent functionalisation are reviewed. A brief illustration of useful recognition methods is also described focussing on the relation of nanotubes with biological molecule. The biocompatibility of carbon nanotubes was investigated as a main requirement for their medical and biological use. The multiple facets of this issue are underlined, the size and shape matter being the main topic of theses studies dedicated to the investigation of carbon nanotube biocompatibility. Emphasizing new avenues of medicine' future, carbon nanotubes are identified as promising candidates for nanomedicine and regenerative medicine with challenges and new opportunities.

In view to prepare the design of a new formulation the complementar contribution of polymeric components was reviewed in order to identify the characteristics could be exploited in achieving the general aim. The preparation of a PLGA colloidal dispersion by a nanoprecipitation method opens new opportunities for the design of fibers. A brief description of polyvinyl alcohol and its properties explore the capacity of this polymer to play the role of host polymer in coagulation process and further to confer advantageous characteristics of the macroscopic fibers.

The very recent achievements in the formulation of CNT neural biomaterial is presented as a sign of the increasing interest in this field. Finally, a brief introduction of nerve cells and the challenge in nerve regeneration close the chapter.This chapter was decisive for the development of this project taking into consideration the emergence of carbon

nanotubes structure, their fabrication and their investigation for medical use, and particularly, neural applications. Despite this increasing interest, up to today, there is not accepted a proposal for a specific formulation for a neural biomaterial based on carbon nanotubes.

The general goal of this thesis is presented in Chapter 2 along with the strategy of the research work and the specific objectives. The development of this step-wise process evidences the multidisciplinary tasks and the need to converge the main steps in combined efforts toward achieving the main goal of the project.

To achieve this project, many experimental techniques were used to perform the process and the investigation following experimental protocol prepared for each operational step. A summary of the main experimental aspects and techniques is accomplished in Chapter 3 were we present the protocol of each experimet, the materials we use as well as the equipements and their characteristics. The techniques used for the characterisation of fibers are briefly described.

If Chapter 1 is the foundation of this work, Chapter 4 is the core of this manuscript and emphasizes on the creation of a new CNT-biomaterial for neural application. Taking into account the nanoscale features of nanotubes and considering the wet spinning process, we propose a hybrid approach for the design of new biomaterial. This approach guarantees the integration of nanotubes and maximizes the translation of

their intrinsic characteristics in the macroscopic fibers. Each fiber is a combination of dispersed CNTs, a polylactic-co-glycolic acid (PLGA) copolymer and polyvinyl alcohol (PVA). The incorporation of PLGA nanoparticles underpins the fiber's structure, allowing the arrangement of nanotubes in the nanoparticles' lattice. A synergistic contribution of the PLGA and the PVA greatly improves the fabrication process and complements the functionality of CNTs. Adopting this new approach we advantageously elude the covalent chemistry of CNTs. Our concept is based on the development of spinable Nanotube-Sphere Binary Colloidal Systems (NSBCS) for a wet spinning process. Binary colloidal systems contain CNTs dispersed with sodium dodecyl sulphate (SDS) and an aqueous suspension of PLGA nanoparticles combined in a variety of ratios. The efficiency of this method resides in the synergistic effect of spherical nanoparticles and rod-like particles assembled in a binary colloid system which plays a main role in the spinning process. By the scheme of the preparation of the mixture, we visualize the simplicity of the proposed approach and its adaptability. The introduction of nanoparticules makes the difference in this formulation. Their participation is correlated with the confinement effect presented in Fig. 4.5-a, visualising the injection step. The colloidal mixture enhances the spinability and creates the conditions for a new coagulation process as presented in the schema of coagulation. This contribution is reflected in the formation of many different structures although the change in the process parameters is not spectacular. The specificity of morphological characteristics we generate suggests the capacity of the new approach to offer a response in the construction of nanosized structured materials as preferential substrates for neural cells.

By initiating the suspension of PLGA nanoparticles, we tailor the characteristics of new fibers, particularly their biodegradability and their biocompatibility. The results demonstrated the potential of biocompatibility. The growth of cells as visualized by microscopic images demonstrates the effects of these fibers. These qualitative performances are a valuable response to the biocompatibility concern because we are not faced with cytotoxicity effects. This evaluation is supported by the quantitative estimation of cell proliferation which indicates high viability of cultured cells. The formation of neurites and their extension on fiber substrate cultured with neural cells in the absence of NGF is very promising for the future investigation of these biomaterials. It is important to note that the content of nanotubes in the fiber is not the only variable which influences the biocompatible response. For very similar contents of nanotubes the performances in terms of viability are different, suggesting the importance of the fashion in which nanotubes are arranged into the fibers. With regards to biodegradability, although we did not perform a systematic study, the presented results demonstrate the biodegradable behaviour of fibers. We expect a controllable rate and the progressive creation of the porous structure which could have great benefits for cell growth. The arrangement of nanotubes in a network preserves the framework of the fiber and eliminates the risk of release of nanotubes in their nanometric form. We stress that the applicability of the hybrid approach to both the SWNTs and MWNTs is a main asset for the optimization of the process.

The investigation of these new fibers, provide the results that strengthens the ability of the proposed method. The performances of SWNT-fibers in term of mechanical characteristics and viscoelastic behavior demonstrate the capacity to modulate the bulk properties of fibers for small content of CNTs. At the same time a systematic investigation is needed in order to explain the particular behaviour displayed by SW-RG503H-2 fiber containing 30% nanotubes, Fig. 5.4. The visualization of the homogenous phase distribution by AFM analysis, as presented in Fig. 4.2, provides the the information which support efficiency of the hybrid aproach in organizing nanotubes either as individual entities or in very thin bundles. Correlated to the mechanical characteristics, these observations complete the correlation structure-properties. The creation of a particular roughness is important for the contact of fibers with living cells, thus creating spatial cues for cell adhesion and migration. The values of roughness demonstrated the capacity of the formulation to modulate the fiber surface by the variation of the size of PLGA nanoparticles, thus enhancing the cell attachment. The visualization of fiber topography demonstrates the capacity to create topographic characteristics in order to improve the contact of fibers with cells. The viability of fibroblast cells, although is not at the same level as that of neural cells, does not indicate the signs of cytotoxicity and proves the ability of several fibers to support the growth of these cells.

Texturing the fiber surface provides a spatial organization for cells and ensures the exposure of nanotubes on the external surface, thus facilitating their contact with

cells at a molecular level. This distribution of nanotubes is revealed by the surface characteristics. Using high performance technique such as Time of Flight Secondary Ion Mass Spectroscopy (TOF-SIMS) and Atomic Force microscopy (AFM) we investigated the chemistry of surface fibers at the nanometric level. The distribution of the ions of each component of the fiber, such as carbon nanotubes, polylactic-co-glycolic acid and polyvinyl alcohol, suggests a very homogenous surface chemistry.

The originality of the fibers, as presented herein, is relevant for the validity of the new method. Its potential lies in the ability to fine-tune the characteristics of rod-like particles of the original CNT dispersion through their distribution in PLGA pseudolatexes, composed from sphere-like particles. A new behaviour arised due to, the interstitial arrangement of the nanotubes in the colloidal mixture, with positive impact on its spinnability. The CNT-fibers produced have particular characteristics, are biodegradable and offer *in vitro* biocompatible response

CHAPTER 8

CONCLUSION AND PERSPECTIVES

The design, formulation, fabrication and investigation of carbon nanotube-based fibers as potential neural biomaterials, were the main objectives of the present study. A hybrid approach is proposed and it consists in the combination of rod-like particles, such as carbon nanotubes with sphere-like particles, such as PLGA nanoparticles in order to form a colloidal mixture that can be spun by the wet spinning process. Our concept is based on the development of spinable Nanotube-Sphere Binary Colloidal Systems (NSBCS) for a wet spinning process.

The introduction of PLGA nanoparticles makes the difference in this formulation. Their participation is rrelated to spatial confinement effects thus enhancing the spinability of the mixture. This contribution is reflected in the formation of many different structures although the change in the spinning process parameters is not spectacular.

By proposing a hybrid approach we introduce a personalised formulation which allows generating fibers with specific characteristics. New fibers containing carbon nanotubes (CNTs), polylactic-co-glycolic acid (PLGA) and polyvinyl alcohol (PVA) in various proportions, are fabricated as substrate for cells.

The validation of this new method is confirmed by its applicability for both single wall carbon nanotubes (SWNTs) and multiwall carbon nanotubes (MWNTs) along with multiple possibilities in variation of the PLGA content. The multiple formulations we realised prove the applicability of the method.

The investigation of fibers conducted to the consistent results in term of bulk composition. The values determined by TGA, prove the reproducibility of the formulation. The mechanical properties, as measured for SWNT-fibers and MWNT-fibers, prove very good mechanical resistance (150 MPa), high elastic modulus (20 GPa) and good elongation (5%). Reported to the content in nanotubes, these values are very significant.

Introduction of PLGA, an amorphous copolymer at room temperature, influences the dynamical mechanical properties and enhances the viscoelatic behaviour of certain fibers, particularly at a temperature close to that of the human body. The fibers display high flexibility with great effects when in direct contact with living cells.

The distribution of three components, CNT, PLGA and PVA, in the fiber and on their surface, along and across the fiber, is very important. The microscopic investigation at nano- and micro-level proves the homogeneity of phase distribution. The AFM images in phase mode are powerful proof for this characteristic. The chemistry at the molecular level, as determined by TOF-SIMS analysis, indicates homogeneity. At the same time, the formation of sub microscopic domains generates periodic structures and textures with great benefits for cell attachment.

Accomplishments in fiber biodegradability and biocompatibility have been demonstrated. When these fibers are cultured with neural cells and fibroblasts, they do not elicit any signs of cytotoxicity. Moreover, the proliferation of neural cells indicate that the CNT fibers are very promising substrates for cells. The formation of neurites and their extension, even in the absence of NGF, sustains the capacity of fibers to stimulate the differentiation of cells.

To conclude, new CNT-based fibers were produced by a hybrid approach based on the development of spinable Nanotube-Sphere Binary Colloidal Systems (NSBCS) for a wet spinning process, and they display unique characteristics. The proposed NSBCS concept proved its feasibility.

The perspectives for pursuing of this study mainly rely on the evaluation of the fibers in order to describe each specific behaviour. More measurements were performed for the investigation of these fibers in order to establish the contribution of each characteristic to the overall performance. The results on electrochemical behaviour should be analysed and reported in order to evaluate the capacitor quality of fibers. As the electrochemical measurements were performed in both the culture medium and solution of dilute acid, this part will provide more information about the electro active behaviour of fibers and its impact on the biocompatibility. The data on quantitative analysis of surface chemistry have to be analysed in order to discern on the role of each chemical formulation in biocompatibility of the fibers.

A systematic study of biodegradable behaviour has to be continued in order to distinguish the contribution of each factor playing a role in this behaviour. A quantitative study for the biocompatibility is needed in order to evaluate the performance of fibers for a long time period and different neural cells should be tested.

Stem cells offer a new alternative to regenerative therapies for neural cells. It would be useful to test these fibers in contact with stem cells.

These CNT fibers have to be investigated through an in vivo model, particularly in the case of spinal cord injuries.

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