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Effect of CNT on Collagen Fiber Structure, Stiffness Assembly Kinetics and Stem Cell Differentiation

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Abstract

Collagen is a native one-dimensional nanomaterial. Carbon nanotube (CNT) was found to interface with biological materials and show promising applications in creating reinforced scaffolds for tissue engineering and regenerative medicine. In this study, we examined the unique role of CNT in collagen fiber structure, mechanical strength and assembly kinetics. The results imply that CNT interacts with collagen at the molecular level. It relaxes the helical coil of collagen fibrils and has the effect of flattening the fibers leading to the elongation of D-period, the characteristic banding feature of collagen fibers. The surface charge of oxidized CNT leads to enhanced local ionic strength during collagen fibrillogenesis, accounting for the slower kinetics of collagen-CNT (COL-CNT) fiber assembly and the formation of thicker fibers. Due to the rigidity of CNT, the addition of CNT increases the fiber stiffness significantly. When applied as a matrix for human decidua parietalis placental stem cells (hdpSCs) differentiation, COL-CNT was found to support fast and efficient neural differentiation ascribed to the elongated D-period. These results highlight the superiority of CNT to modulate collagen fiber assembly at the molecular level. The study also exemplifies the use of CNT to enhance the functionality of collagen for biological and biomedical applications.

Keywords

Collagen; CNT; fiber assembly; D-period; stem cell neural differentiation

1. Introduction

As a biomaterial, collagen is highly robust. It has been frequently used as a cell culture matrix, introduced to medical devices, and applied in tissue engineering and material science [1-3]. Among the various types, collagen type I is the main supportive protein within fascia, cartilage, ligaments, tendons, bone, skin and other connective tissues, due to its great tensile strength [4, 5]. The unique viscoelastic and mechanical properties contribute to considerable energy dissipation capacity of collagen [6, 7]. The biochemical process behind collagen's

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structure and self-assembly has been extensively studied for several decades. Collagen type I is composed of three polypeptide chains that interact to form a single right-handed super helix, the monomeric form of collagen. Each monomer is ~300 nm in length and 1.5 nm in diameter [8]. Five triple-helical collagen molecules, arranged within the fibril with quasi-hexagonal packing, form a collagen microfibril with the characteristic 67 nm banding feature, the D-period. The D-period itself has overlap and gap regions, with five monomers in the overlap and only four in the gap region. It was reported that collagen D-period varies (typically 2-4 nm) in response to strain and dehydration [9-11]. This is ascribed to the combination of molecular elongation, an increase in the gap region between longitudinally adjoining molecules along the fiber axis, and the relative slippage of laterally adjoining molecules along the fiber axis [9, 12, 13]. A noticeable change of 2.5 nm in D-period was found in Osteoporosis [14], and the change was ascribed to collagen type I mutation which causes conformational changes in triple helix and consequently in collagen fibril assembly [15, 16]. Thus, a change in D-period denotes the variation in collagen molecular assembly.

Collagen fiber assembly is a stepwise process, involving the initiation of microfibril formation, the linear growth of fibrils, and the lateral association of fibrils into a network [17-19]. At the macroscopic scale, collagen type I forms a network of interconnected fibers upon gelation at 37 °C. Collagen type I is quite amenable to manipulation. It has been reported that nanostructure and elasticity of the fibers can be modulated by varying temperature, pH, ionic strength and collagen concentration [20, 21]. In our previous studies, we have incorporated carbon nanotubes (CNTs) into collagen type I (COL-CNT), and found the COL-CNT matrix induced accelerated neural differentiation of both embryonic stem cells [22] and adult stem cells [23] when compared to a pure collagen matrix. Others also reported that the addition of CNT to collagen resulted in increment of cell adhesion compared to collagen-coated dish [24]. Hydrogel matrix prepared by polymerizing COL-CNT with human dermal fibroblast cells was found to reduce compaction over time when compared with matrices without CNT [25]. When exposed to electrical stimulation, COL-CNT facilitated the differentiation of PC-12 cells into neurons [26]. These studies suggest the potential application of COL-CNT in tissue engineering, and that the addition of CNT is a viable means for manipulating collagen's properties for the purpose of directing cell fates.

While it is clear that COL-CNT scaffolds are highly useful substrates, very little is known about the mechanism of collagen-CNT interaction and what unique role that CNT plays in such composite materials. In this work, we aim at deliberating the interaction by probing the nanoscale structural and physical differences between collagen and COL-CNT. With results derived from atomic force microscopic (AFM) measurements, kinetic study of fibrillogenesis and optical rotation, we postulate a mechanism for the intrinsic collagen-CNT molecular interaction in collagen fiber assembly. The contribution of CNT to accelerated commitment and maturation of human decidua parietalis stem cells (hdpPSCs) to neural cells was found to be attributable to the altered D-period in the collagen-CNT fibers.

2. Materials and Methods

2.1. Functionalization of CNT

High purity single-walled carbon nanotube (SWCNT) was purchased from HELIX Material Solution (Richardson, TX). Raw SWCNTs were functionalized with carboxyl group by sonicating 10 mg of SWCNT in a solution of 8 M of H_2SO_4 and 8 M of HNO_3 at 70°C for 3 hours. The SWCNT solution was then centrifuged at 1398 g force for 10 min., and the supernatant was collected and diluted with Milli Q water (18.2 M Ω). The oxidized SWCNT was filtered by using a glass microanalysis filter system (Millipore, Billerica, MA) with 0.22 μm isopore membranes (Millipore, Billerica, MA). The collected CNT on the membrane was redispersed in Milli Q water. The procedure was repeated until a pH of 7 was achieved [27, 28]. The oxidized CNT was collected as powder after drying in a vacuum oven overnight at 70°C . The successful oxidation of CNT was verified by infrared (IR) peaks at around 1700 cm^{-1} (carbonyl group) and 3400 cm^{-1} (hydroxyl group), which are absent in raw CNT.

To examine the co-localization of CNT and collagen, CNT was labeled with a fluorescent dye, fluorescein isothiocyanate (FITC, Sigma-Aldrich, St. Louis, MO). The reaction of amine-terminated FITC and oxidized CNT was catalyzed by 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) in 0.1 M 2-(N-Morpholino) ethanesulfonic acid hydrate (MES) buffer (pH=6). The dye-labeled CNT was similarly collected and dried as oxidized CNT was. The amide bond formation was confirmed by the appearance of the IR peaks at 1636 cm^{-1} and 1458 cm^{-1} for amide I and amide II groups, respectively.

2.2. Preparation of matrices

Collagen type I from rat-tail was purchased from BD Biosciences (Bedford, MA). 1.5 mg/ml collagen solution was made according to the manufacturer's protocol. Sterile tissue culture dishes were coated with this solution and incubated at 37°C overnight. COL-CNT solution was prepared by adding 0.1, 0.25, 0.5 and 0.7 w% of oxidized CNT (with respect to collagen weight) to 1.5 mg/ml collagen solution. Sterile tissue culture dishes were incubated with this solution at 37°C overnight to form the COL-CNT matrix. Collagen- TiO_2 (COL- TiO_2) matrix was also prepared by adding TiO_2 nanotubes to 1.5 mg/ml collagen solution for control studies. TiO_2 nanotube powder was obtained as a gift from Dr. Kazuya Nakata at Kanagawa Academy of Science and Technology, Japan. To study the effect of ionic strength on collagen fiber formation and fiber mechanics, NaCl was added to 1.5 mg/ml collagen solution to prepare the collagen-NaCl (COL-NaCl) matrix. As another control, gelatin coated substrates were prepared by incubating the tissue culture dishes overnight with commercially available 0.1% gelatin (Fisher scientific, Fairlawn, NJ).

2.3. Atomic force microscopic imaging and fiber stiffness measurement

Both AFM imaging and stiffness measurements were carried out in PBS buffer using Si_3N_4 tips with a spring constant of $0.056 \pm 0.002\text{ N/m}$, calibrated by using reference cantilevers with known spring constants [29-32]. A multimode Nanoscope IIIa AFM (Veeco Metrology, Santa Barbara, CA), equipped with a J-scanner, was utilized in this study. Imaging was

performed in fluid tapping mode, with topographic and amplitude images captured simultaneously. The fiber stiffness measurements were conducted by adopting the nano-indentation method, in which an AFM tip was employed as a nano-indenter to probe a one-dimensional material. From the generated force spectra, the young's modulus, E , was derived when the following equation was applied [33]:

$$E = \sqrt{\frac{9F^2}{16R_c\delta^3}} \text{ where } R_e = \sqrt{\frac{R_t^2 R_c}{R_t + R_c}} \quad (1)$$

E is the Young's modulus, F is the applied force, δ is the nano-indentation depth and R_e is the equivalent radius. R_t is the radius of the tip and R_c is the radius of a 1D material, such as a collagen fiber. The elasticity of a sample varies with the nano-indentation depth ($\delta = z - d$) [29]. Here we chose an indentation depth of 25 ± 8 nm, corresponding to the linear range of the force curves, to evaluate the Young's modulus. We previously derived the E values from the initial rise of the force curves [23]. The two sets of data are different, however the trends of changes are consistent. The average E was calculated from E -maps consisting of 32×32 data points in $4 \times 4 \mu\text{m}^2$ images. Measurements were made from at least 20 areas in different samples using the same or different tips.

2.4. Kinetics of collagen fibril assembly

To measure the kinetics of collagen fibrillogenesis, we carried out turbidity tests by collecting the time-dependent optical absorbance at 313 nm using a CARY 500 scan Varian UV-Vis-NIR spectrophotometer (Agilent Technologies, Santa Clara, CA). Gelation of pure collagen, COL-CNT, COL-TiO₂ and COL-NaCl were examined under comparable conditions in parallel studies. In order to evaluate the rate of fiber assembly, we derived the initiation time (t_i), defined as the time at which the absorbance started to rise, and the second-phase halftime ($t_{II, 1/2}$), defined as the time at which the absorbance reached half of its final value [20]. The final absorbance is the absorbance at the steady state.

2.5. Specific rotation measurement

A JASCO P-2000 polarimeter (JASCO Inc, Easton, MD) was used to examine the specific rotation of the fibers of pure collagen, COL-CNT and COL-TiO₂ at 589 nm. The pre-mixed solution was transferred to a cylindrical glass cell (JASCO CG3-100), which was mounted in the sample chamber. The specific rotation data were collected at one-minute intervals for about one hour until the readings became steady. This steady-state value was recorded as the specific rotation of the mature fibers. Statistical analysis was performed based on 20 separate measurements.

2.6. Cell Culture

Undifferentiated hdpPSC cells, isolated from the decidua parietalis dissected from human term placenta, was kindly provided by Dr. Strakova's lab at University of Illinois at Chicago, and propagated in a self-renewal media according to the pre-defined protocol [34]. For differentiation experiments, the undifferentiated cells at passage 3-5 were trypsinized and

plated on various matrices in the non-selective, spontaneous differentiation medium (DMEM + 10% FBS+ 1% non-essential amino acids) at a density of 500 cells/cm² per matrix.

2.7. Cell proliferation assay and cell viability test

The cell proliferation was evaluated by counting the number of live cells at different time points. A 0.25% trypsin EDTA (Mediatech Inc., Manassas, VA) was added to cell dishes. Detached cells were collected three minutes later. 10 µl of the cell solution was added to a hemacytometer and the total cell number in the grid was counted under a TE-U 2000 Nikon microscope. Filtered trypan blue solution of 0.4 % (w/v) in PBS (Mediatech Inc, Manassas, VA) was used to stain the non-viable cells in the cell viability test. The percentage of viable cells was quantified based on the number of trypan blue-staining cells and the total number of cells.

2.8. Immunofluorescence staining

The expression of neuronal markers, Tuj-1 and Neu-N, was tracked by immunofluorescence staining at Day 1, 3 and 6 of differentiation using a Nikon U-2000 microscope. The primary antibodies used in this study include rabbit anti-Tuj-1 (β-3 tubulin, Abcam, Cambridge, MA, 1:200 dilution) and mouse anti-NeuN (Millipore, Temecula, CA, 1:100 dilution). Secondary antibodies with fluorescent tags of Alexa Fluor 594 and Alexa Fluor 488 were purchased from Invitrogen (Carlsbad, CA) and used at a dilution of 1:200. The exposure time and gain value were kept constant for each marker across all the samples and time points, and a negative control was performed by excluding the primary antibody during the staining protocol for each set of experiments. The mean intensity in these negative control samples was essentially zero. The immunofluorescent images were quantitatively analyzed by ImageJ software for determining the relative expression levels. Image size, magnification and imaging parameters were maintained constant throughout all measurements to avoid variations between samples. In determining the percentage of positively stained cells within an image, a cell was considered positive when its intensity was at least two folds or more above the background fluorescent intensity. The number of cells were manually estimated from images captured using 20× objective of the microscope. Images were collected on samples prepared from a minimum of six experiments, two identical samples per experiment, six regions per sample. ImageJ analysis was performed on at least 360 cells for each matrix type for statistical analysis.

3. Results

3.1. Structure, stiffness and assembly kinetics of collagen and collagen-composite fibers

3.1.1. Nanoscopic structures—To examine the distinct nanoscopic features of collagen, COL-CNT and other composite fibers, high-resolution AFM imaging was carried out. Fig. 1A shows an AFM image of SWCNTs deposited on a Si substrate. The tubes are as thin as 1.2 nm in diameter. They are hardly visible in images of COL-CNT fibers (Fig. 1C, D). Nevertheless, their effect is evident. Pure collagen fibers (Fig. 1B) are thin (53 nm in diameter), well spread across the surface (higher surface coverage), and flexible. In contrast, the COL-CNT fibers are thicker and more rigid. When the CNT percentage increases, fibers are getting thicker and more localized with less surface coverage. The addition of CNT to

collagen also caused the increase of the D-period. As summarized in Table 1, while a 0.1 w % CNT addition induced a 1 nm increase of collagen D-period, a 0.25 w % CNT addition caused a significant 3 nm increase ($p < 0.0001$). CNT addition of more than 0.25 w % didn't lead to further increase in D-period, however the fiber width displayed broader distribution likely due to the high level “impurity” that disrupted fiber assembly. Thus, 0.25 w % CNT was considered the optimum amount of CNT addition to induce coherent changes in collagen fiber structure under the current fibrillogenesis condition, and was used throughout the rest experiments. Given that there are about 150 carbon atoms per 1 nm length of SWCNT [35], a 0.25 w % of CNT in collagen represents an approximate ratio of 1:1000 between the equivalent length of CNT nanotubes and collagen molecules (tropocollagen).

Carboxyl-functionalized CNT has a negatively charged surface, thus may increase the local ionic strength when it is brought into close interaction with collagen fibers and is accountable for the increased fiber width [20, 36, 37]. This was attested by examining collagen fiber formation in a gelation medium with an increased ionic strength. The increase in fiber width with the addition of NaCl in the gelation medium was observed. When 60 μ M NaCl was added, the COL-NaCl fibers (Fig. 1E) were at a similar dimension (~ 260 nm in diameter) as those of COL-CNT at 0.25 w % CNT. Despite the increase in medium ionic strength, the D-period remained at 67 ± 1 nm in COL-NaCl fibers. However, these fibers show variable width along the fiber axis, and they irregularly branch and cross each other (Fig. 1E). Apparently, the incorporation of CNT to collagen occurs in a more uniform way, leading to coherent changes during fiber assembly, hence, forming uniform fibers.

3.1.2. Fiber stiffness—With the increase in CNT percentage, COL-CNT fibers appeared thicker and less flexible. The fiber stiffness was evaluated by the nano-indentation method. As shown in Table 1, the stiffness of collagen fibers increases with the percentage of CNT. While the addition of NaCl in the medium also causes the increase in fiber stiffness, when fibers with similar width are compared, COL-CNT fibers are much stiffer than COL-NaCl fibers. As a control, TiO₂ nanotube was applied as an alternative to CNT. As illustrated in Fig. 1F and Table 1, an addition of 0.2 w % TiO₂ nanotube to collagen increased the fiber stiffness and width to the same levels as the addition of 0.25 w % CNT did, and noticeably, the D-period remained unchanged at 67 ± 2 nm even the percentage of TiO₂ nanotube was further increased.

3.1.3. Fiber assembly kinetics—A fiber structure is relevant to the fiber assembly kinetics. The kinetics of collagen, COL-CNT (0.25 w %), COL-NaCl (60 μ M) and COL-TiO₂ (0.2 w %) fiber assembly was examined by measuring the solution turbidity (absorbance) at 315 nm [17, 20]. The solutions contain the same concentration of collagen (1.5 mg/ml), and the resulted collagen-composite fibers have either similar stiffness (COL-CNT and COL-TiO₂) or similar dimension (COL-CNT, COL-NaCl and COL-TiO₂). As shown in Fig. 2A, all curves show sigmoidal characteristics, consistent with the two-phase cooperative mechanism of collagen fibrillogenesis originally proposed by Wood *et al* [38]. The solution is transparent at the initial phase of fiber assembly, which involves the aggregation and stacking of monomeric collagen to form short microfibrils. This was evidenced by the appearance of thin fibrils of 1.5 nm to 6.0 nm in diameter and 300 nm to

800 nm long in AFM images captured within 5 minutes of fiber gelation. It suggests the presence of both monomeric collagen (~1.5 nm thick) and the rapidly forming microfibrils (~6 nm thick) at this stage. In the second phase, the microfibrils further assemble laterally and longitudinally and develop into thick and long fibers exhibiting the characteristic D-period banding pattern. This phase is characterized by a fast increase in solution turbidity until the thermodynamic equilibrium is reached. According to curves in Fig. 2A, we derived the initiation time (t_I) and the second-phase halftime ($t_{II,1/2}$) of fiber assembly, and concluded them in Fig. 2B. In both phases, collagen-composites took longer time than pure collagen to assemble. Interestingly, $t_{II,1/2}$ of COL-TiO₂ was shorter than that of COL-CNT, though it was longer than that of pure collagen. COL-NaCl showed dramatic delay of fiber assembly in both phases when compared to other fiber types, likely due to the high ionic strength in the bulk solution that tends to preserve the system at a near-steady state [39]. Note that the final absorbance of the collagen matrix is higher than that of the other matrices, consistent with the high surface coverage of thin collagen fibers as observed in the AFM image (Fig. 1B).

Taken together, incorporation of CNT into collagen led to the increase in collagen fiber width and stiffness, the increase in collagen D-period and the delay of fiber assembly. While collagen fiber width and stiffness can be altered by varying the ionic strength of gelation medium or by incorporating alternative nanotubes, statistically significant change in D-period was achieved only by adding CNT to collagen.

3.2. Collagen-CNT interaction

3.2.1. Location of CNT in collagen fiber network—To elucidate how CNT interacts with collagen fibers, the relative location of CNT with respect to collagen molecules was examined. CNT was labeled with FITC in order to analyze by fluorescence imaging. From Fig. 3 (A) and (C), it is evident that COL-CNT fibers are thicker than pure collagen fibers, consistent with the results from AFM studies (Fig. 1 and Table 1). With pure collagen as a negative control (Fig. 3C, D), CNTs are shown to co-localize with collagen fibers (Fig. 3A, B), instead of spreading all over the substrate. CNTs either reside physically within a single collagen fiber or are trapped between fibers. We speculate that CNT is neatly integrated into collagen fibrils to induce the change in collagen D-period during fiber assembly.

3.2.2. Changes in optical rotation—Collagen is made up of left-handed helical protein strands of α -1 and α -2 chains and form right-handed super helix that has a specific optical rotation. A molecular level interaction between collagen and CNT is expected to affect the specific rotation. The specific rotation measured in pure collagen is $-330 \pm 19^\circ$, consistent with that reported by others [40]. The specific rotation measured in COL-CNT is $-303 \pm 16^\circ$, and the difference with respect to that of pure collagen is statistically significant ($p < 0.0001$). As a control, the specific rotation in COL-TiO₂ was also measured, and the value of $-328 \pm 23^\circ$ is not significantly different from that of pure collagen ($p = 0.77$). This supports our hypothesis that CNT uniquely participates in collagen molecular assembly.

3.2.3. Evidence from high-resolution imaging—By high-resolution AFM imaging, the relative height between the overlap and the gap regions (referred as the apex) of a D-period was quantified in collagen and COL-CNT fibers as illustrated in Fig. 4 A and C. We

found the apex was lowered from 4.3 nm in pure collagen to 3.4 nm in COL-CNT. The apex was essentially unchanged (4.1 nm) in COL-TiO₂. Taken together with the optical rotation data, it appears that CNTs flatten the collagen fibers axially and extend the fiber D-period.

3.3. Application of COL-CNT in hdpPSC differentiation

3.3.1. Effect of COL-CNT on hdpPSC proliferation and viability—In order to test if CNT affects cell proliferation, the cell density was evaluated on pure collagen, COL-CNT and gelatin from Day 1 to Day 6 (Fig. 5A). On Day 1, the cell density on collagen and COL-CNT was lower than that on gelatin. This is mainly due to the difference in matrix adhesion for initial cell attachment. With the increase of the culture time, the rates of cell proliferation on collagen, collagen-CNT were comparable to that on gelatin. When the growth rate was evaluated at the logarithmic phase, the doubling time of hdpPSCs was found to be in a narrow range: 52 h, 47 h and 56 h for cells on collagen, COL-CNT and gelatin, respectively. Cell proliferation on COL-CNT with varied CNT concentration was also examined. When CNT concentration varied between 0.1 w% and 0.7 w%, no significant difference in cell proliferation was observed on these matrices when compared with that on pure collagen (Fig. S1). Thus, the presence of CNT in the COL-CNT matrix does not impede cell proliferation, at least in the concentration range of 0.1 w% to 0.7 w%.

The cell viability was also tested on different matrices (Fig. 5B). From Day 1 to Day 6, the cell viability on gelatin was near 100 %. The cell viability on Day 1 on collagen and collagen-CNT was slightly lower (95 ~97%); however, no statistically significant difference was observed between cells on collagen and COL-CNT ($p=0.45$ on Day 1). The cell viability increased later and reached more than 99% on Day 6.

Taken together, the nature of a matrix indeed affects cell proliferation at the initial stage. However, cells adapt the new environment in later days, and proliferate and remain viable at a comparably high rate on all matrices.

3.3.2. Effect of COL-CNT on hdpPSC differentiation—To examine the matrix effect on stem cell differentiation, the hdpPSC differentiation profile on collagen, COL-CNT (0.25 w%), COL-TiO₂ (0.2 w%), COL-NaCl (60 μ M) and gelatin (a featureless substrate) was compared under the non-specific differentiation (spontaneous differentiation) condition. Cells were grown on the matrices for 6 days and were stained against the neural markers, Tuj-1 and NeuN, on Day 1, Day 3 and Day 6 of differentiation. From the immunofluorescence images of cells on Day 6 of differentiation (Fig. 6A-E), it is clear that the bipolar morphology of cells is common on collagen, COL-CNT, COL-TiO₂ and COL-NaCl matrices, but is rarely seen on gelatin. However, cells resembling typical neural cells, exhibiting a small cell body, branched dendrites, and a long filament-like extension (white arrows in Fig. 6C), are highly dominant only on the COL-CNT matrix. Additionally, cells on COL-CNT stained both Tuj-1 (green) and NeuN (red) at substantially higher intensity than on any other matrices. The percentage of NeuN and Tuj-1 positive cells on Day 1, Day 3 and Day 6 of differentiation for all the matrices was further quantified, and the data are summarized in Fig. 6 F and G. On gelatin, the expression levels of Tuj-1 and NeuN were quite low even until Day 6, indicating the neural differentiation was deterred on gelatin

matrix. On collagen, only a small percentage (5%) of cells were Tuj-1 positive by Day 3. However, by Day 6, neural differentiation seemed to be progressing since roughly 40% of the cell population were Tuj-1 and NeuN positive. Neither COL-TiO₂ nor COL-NaCl outperformed collagen in supporting the neural differentiation of hdpPSCs. In contrast, more than 60% of the cells on COL-CNT were already positive for both Tuj-1 and NeuN by Day 3 of differentiation. By Day 6, majority of the population (over 80% of cells) expressed both markers at high levels, and particularly, 92% cells were NeuN positive. The statistical analysis by a t-test suggests that on both Day 3 and Day 6, the expression of NeuN or Tuj-1 on a COL-CNT matrix is different significantly from that on the rest matrices ($p < 0.005$). The result implies that the COL-CNT matrix is capable of supporting the development of mature neural cells at a faster pace.

4. Discussion

The ability to alter collagen's properties by incorporation of extraneous agents offers a simple, robust yet mild approach for manipulating collagen's properties while retaining its inherent biocompatibility. It was reported that the width and mechanical strength of collagen fibers are susceptible to changes in buffer pH [41], salt concentration [42] and hydration level of the fiber environment [6]. However, in these studies, either very high concentration (6 mg/ml) of collagen or very harsh conditions (acetic acid or ethanol) were used. In the current study, all matrices were prepared at the same collagen concentration (1.5 mg/ml) in the same buffer at a pH of 7.4. The addition of a minute amount of CNT resulted in discernible alterations in collagen fiber thickness and stiffness. Addition of TiO₂ nanotubes to collagen was able to induce similar results. The enhanced fiber width and stiffness can also be mimicked by the addition of NaCl to increase the bulk ionic strength of the gelation solution, leading to our speculation that CNT and TiO₂ nanotubes may mediate collagen-collagen association by a similar mechanism- an increase in the local ionic strength. The assumption is well-grounded when considering that the carboxyl groups on an oxidized CNT and the hydroxyl groups on a hydrolyzed TiO₂ nanotube provide the negative surface charges to draw collagen molecules close and increase the local ionic strength in the vicinity. This in turn reduces the electrostatic interaction between neighboring collagen strands to promote a tighter fibril-fibril association [36]. It leads to concomitant increase in fiber width and fiber stiffness, as shown in Table 1. The increase in collagen fiber stiffness by increasing the media ionic strength alone is at a much less extent than by adding CNT or TiO₂ nanotubes. Apparently, the integration of rigid nanotubes greatly improves the compression resistance of the flexible collagen fibers. The increase in fiber width, relevant to a higher local ionic strength, causes a tighter bundling of fibrils to enhance the fiber strength, but plays a minor role in fiber stiffness.

The extended lateral growth of collagen-composite fibers resulted in a slower fibrillogenesis rate at both the initial phase and the second phase of fiber assembly (Fig. 2). Interestingly, the addition of CNT reduced the rate of collagen microfibril assembly more significantly than the addition of TiO₂ nanotube, and CNT only induced the increase in fiber D-period. The data suggests that the addition of CNT not only causes the extensive lateral growth of fibers, but also results in the longitudinal fibril development through a characteristic CNT-collagen molecular interaction. Thus, we hypothesize the model illustrated in Fig. 4B to

interpret the effect of CNT on collagen fiber assembly. Due to its high rigidity, CNT likely aligns along the side of the collagen molecules. The enhanced local ionic strength, due to the presence of oxidized CNT, facilitates the lateral assembly of collagen fibrils to form thick collagen fibers. The insertion of CNT among the collagen molecules has the effect of compressing the fibers in the direction perpendicular to fiber axis, or in other words, flattening the molecular “crinkles” of the fibers longitudinally, causing the elongation of the D-period and consequently the decrease of the apex (*i.e.*, the overlap-gap height). Note that the average length of oxidized CNT is around 300-600 nm (upon sonication), and the length of an individual collagen monomeric molecule is 300 nm. The width of single-walled CNT (1.2 nm) is similar to that of molecular collagen (1.5 nm). While negatively charged carboxyl groups are on the surface of oxidized CNT, positively charged amine groups are available at the exterior of collagen fibers. The similar dimension of the two one-dimensional materials and the opposite charges available on the surfaces likely draw CNTs and collagen molecules close enough to establish hydrogen bonding and electrostatic interaction that play critical roles in stabilizing the entropically unfavorable collagen-CNT alignment with a longer D-period. Within the overlap region, as shown in Fig. 4, collagen molecules are tilted approximately 15° with respect to the fiber axis [43]. The decrease in the apex is expected to reduce the tilting angle of the molecules, leading to slightly unwound fibers. This agrees with the observed decrease in specific rotation in collagen-CNT fibers (Fig. 4C), which suggests that the addition of CNT relaxes the helical coil of the assembled collagen molecules.

The collagen-TiO₂ nanotube interaction is evidently different from the interaction between collagen and single-walled CNT (SWCNT). Due to the relatively large diameter of a TiO₂ nanotube (~20 nm), steric hindrance likely prevents it from intimately interacting with collagen in the same way as SWCNT. Screening of more nanomaterials, including multi-walled CNT, is expected to reveal the criteria of forming stable and uniform collagen-composite fibers with tunable D-period, which will provide unique molecular cues in biological applications.

One application demonstrated here is to use COL-CNT as a matrix for the culture of sensitive cells, namely stem cells [44-46]. Fibrillar collagen type I has been routinely used as a matrix component in culturing neural stem cells or neural progenitors due to integrin-mediated intracellular signaling [44, 46-48]. Our results show that the COL-CNT matrix is as conducive as pure collagen to hdpPSC proliferation, and the presence of CNT in the collagen matrix does not affect cell viability. In our previous studies, it was found that the COL-CNT matrix induced accelerated neural differentiation of both embryonic stem cells [22] and adult stem cells [23] when compared to a pure collagen matrix. In the current study of hdpPSCs differentiation on COL-CNT and other collagen-composite matrices, COL-CNT was found the most effective matrix for rapid neuronal differentiation. It should be noted that stem cells differentiate randomly even on a featureless substrate in a non-differentiation (self-renewal) medium, and the differentiation is slow since the medium is not conducive for cell differentiation [49-51]. Consistent results were obtained in our comparative study of hdpPSC culture in non-differentiation and differentiation media (Fig. S2). In the non-differentiation medium, the differentiated cells did tend to select the neural lineage on collagen and COL-CNT; however, no significant difference in differentiation profile was

observed between cells on the two matrices. The use of the spontaneous differentiation medium (non-selective differentiation medium) greatly catalyzed the differentiation of cells on all matrices. Hence, the COL-CNT matrix and the differentiation medium played a concerted role in elevating hdpPSC differentiation toward the neural lineage.

Like the COL-CNT matrix, the COL-TiO₂ and COL-NaCl matrices also exhibit stiffer and thicker fibers. The striking difference, however, is the longer D-period in COL-CNT fibers. Given that only on the COL-CNT matrix, the neuronal phenotype was dominant and the differentiation was established faster, the structural and biochemical cues associated with the elongated D-period are likely more critical than other physical properties, *i.e.* fiber stiffness or fiber width, in promoting the neural differentiation of hdpPSCs. This assumption is consistent with many other reports, which suggested that the specific organization, composition and presentation of ligands exhibited by the extracellular matrix can direct stem cell differentiation. For instance, Lee *et al.* reported that controlling ligand composition and deposition density can coax the neurogenesis of mesenchymal stem cells [52]. By changing the lateral spacing of RGD peptide, Frith JE *et al.* were able to switch the cell fate between adipogenic and osteogenic lineages in mesenchymal stem cell differentiation [53]. In the case of collagen type I, GFPGER⁵⁰²⁻⁵⁰⁷ is the specific ligand sequence responsible for β -1 integrin binding. This sequence is present in the cell-matrix interaction domain at the overlap region of the collagen D-period [54]. Our speculation that the elongated D-period may have direct implications on the β -1 integrin-mediated neural differentiation is based on the following rationale. A 3 nm increase in collagen D-period leads to a significant increase in the ligand spacing, and the alignment of CNT along the side of tropocollagen hinders the accessibility of the amino acids within the β -1 integrin-binding region (*i.e.* GFPGER⁵⁰²⁻⁵⁰⁷). Upon cell adhesion to the COL-CNT matrix, this change in ligand presentation affects integrin expression on cells, and subsequently the formation of focal adhesion complexes, the actomyosin contractility and the downstream integrin-linked signaling cascades. This is consistent with the results from our previous biochemistry study [23] that hdpPSCs expressed β -1 integrin at a lower level on COL-CNT than on pure collagen, and the low β -1 integrin expression mediated a mechanotransduction pathway that imposed the accelerated neural fate. The reduced β -1 integrin expression level in cells on COL-CNT supports the notion that the elongated D-period and the CNT alignment alongside of collagen can modulate the expression of β -1 integrin, hence, the subsequent signal transduction. In summary, our findings add further evidence to the important role of matrix ligand profile on stem cell differentiation, as reported by others as well [53, 55, 56].

5. Conclusion

In this study, we report on the manipulation of collagen's packing structure with simple, mild and efficient means that can potentially provide therapeutic benefits. In particular, the unique role of CNT in altering collagen fiber structure and mechanics during the fiber assembly was identified, and a model to depict the molecular interaction between collagen and CNT was proposed. We provided evidences of the biocompatibility and the promising functionality of COL-CNT in effectively mediating the neural differentiation of stem cells associated with the elongated D-period. The study has two major implications: 1) our results provide insights into the fibrillogenesis of collagen-composite materials; 2) COL-CNT is a novel and

unique bio-composite material that can generate neural cells with a yield as high as 92% in the absence of pro-neural media supplements. This matrix has great potentials in serving as a scaffold for stem cell implantation in applications of developing mature neurons at the site of injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The effect of CNT on collagen fiber structure, stiffness and assembly was examined.
- The changes were attributed to increased local ionic strength and CNT rigidity.
- The molecular interaction between collagen and CNT induced the D-period elongation.
- COL-CNT matrix provides unique cues to promote neural differentiation.

Fig. 1. AFM amplitude images of CNT and collagen-composite fibers. A) CNT; B) collagen fibers; C) COL-CNT fibers with 0.1 w% CNT; D) COL-CNT fibers with 0.25 w% CNT; E) COL-NaCl fibers with 60 μM NaCl in gelation medium; F) COL-TiO₂ fibers with 0.2 w% TiO₂ nano-tubes. The inserts of B and D are the $1 \times 1 \mu\text{m}^2$ higher resolution images illustrating the characteristic D-period.

B

	Collagen	COL-CNT	COL-NaCl	COL-TiO ₂
t_i (min)	4 ± 2	9 ± 2	15 ± 2	8 ± 3
$t_{II,1/2}$ (min)	3 ± 2	8 ± 2	10 ± 2	5 ± 3

Fig. 2.

A) Fiber assembly kinetics of collagen and collagen-composites monitored by measuring the solution turbidity at 315 nm. A constant collagen concentration of 1.5 mg/ml was used in all solutions, and the pH is buffered at 7.4. B) Summary of the initiation time (t_i) and the second-phase halftime ($t_{II,1/2}$) of fibrillogenesis for various matrices.

Fig. 3. Collagen-CNT co-localization examined by optical imaging. A,B) Phase (A) and fluorescence (B) images of the fiber network of COL-CNT. The inset of (B) illustrates the staining of CNT exclusively on a fiber as shown in the merged phase and fluorescence images. C,D) Phase (C) and fluorescence (D) images of the fiber network of pure collagen. Image magnification: 20 $\times$.

Fig. 4. CNT induced nanoscopic alteration of collagen molecular arrangement. A) AFM image of a segment of collagen fiber highlighting the 67 nm D-period; B) Schematic model illustrating CNT alignment with collagen molecules causing the D-period elongation and the apex decrease; C) Summary of the molecular parameters in collagen and COL-CNT.

Fig. 5. Proliferation (A) and cell viability (B) profiles for hdpPSCs on different matrices at Day 1, Day 3 and Day 6 post-plating.

Fig. 6.

Neural differentiation of hdpPSCs on various matrices. A-E) Immunofluorescent images illustrating the costaining of NeuN (red) and Tuj-1 (green) for cells cultured on gelatin (A), collagen (B), COL-CNT (C), COL-TiO₂ (D) and COL-NaCl (E) on Day 6 of differentiation. White arrows in (C) highlight the neuronal progenitor cells. Image magnification: 20 ×. F,G) Bar graphs showing the percentage of Tuj-1 (F) and NeuN (G) positive cells on Day 1, Day 3 and Day 6 of differentiation on various matrices. The data were summarized from immunofluorescent images and quantified by ImageJ analysis.

Comparison of collagen D-period, fiber width and fiber stiffness in collagen and collagen-composite fibers. The D-period and the fiber width were evaluated based on more than 30 measurements for each matrix type and trial. The p values from t-test indicate whether the D-period of collagen-composite fibers is different significantly from that of pure collagen fibers. The matrix stiffness was measured by nano-indentation method, and was derived from 5 different samples, 256 or 512 data points on each sample.

Table 1

	COL	COL-CNT	COL-CNT	COL-CNT	COL-CNT	COL-NaCl	COL-TiO ₂
		0.1 w%	0.25 w%	0.5 w%	0.7 w%		
D-period (nm)	67 ± 2	68 ± 1	70 ± 1	70 ± 1	71 ± 1	67 ± 1	67 ± 2
T-test (p)		0.0005	< 0.0001	< 0.0001	< 0.0001	0.81	0.67
Width (nm)	53 ± 40	100 ± 31	238 ± 30	250 ± 69	265 ± 90	260 ± 28	245 ± 70
Stiffness (MPa)	1.2 ± 0.4	1.8 ± 0.8	3.2 ± 1.5	4.0 ± 1.7	4.3 ± 1.7	2.0 ± 0.4	2.9 ± 1.3