Cellular Uptake of Single-Walled Carbon Nanotubes in 3D Extracellular Matrix-Mimetic Composite Collagen Hydrogels

Article *in* Journal of Nanoscience and Nanotechnology · March 2014

DOI: 10.1166/jnn.2014.8526 · Source: PubMed

CITATIONS
12

READS
607

3 authors, including:

Hongli Mao
Nanjing Tech University
70 PUBLICATIONS **1,528** CITATIONS

SEE PROFILE

Article

Journal of
Nanoscience and Nanotechnology
Vol. 14, 2487–2492, 2014
www.aspbs.com/jnn

Cellular Uptake of Single-Walled Carbon Nanotubes in 3D Extracellular Matrix-Mimetic Composite Collagen Hydrogels

Hongli Mao[1,2], Naoki Kawazoe[1], and Guoping Chen[1,2,*]

[1] *Tissue Regeneration Materials Unit, International Center for Materials Nanoarchitectonics, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan*
[2] *Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan*

Carbon nanotubes (CNTs) exhibit intrinsic unique physical and chemical properties that make them attractive candidates for biological and biomedicine applications. An efficient cellular uptake of CNTs is vital for many of these applications. However, most of the cellular uptake studies have been performed with a two-dimensional cell culture system. In this study, cellular uptake of single-walled carbon nanotubes (SWCNTs) was investigated by using a three-dimensional cell culture system. Bovine articular chondrocytes cultured in SWCNTs/collagen composite hydrogels maintained their proliferation capacity when compared to the culture in collagen hydrogels. Ultraviolet-visible-near-infrared spectroscopy analysis revealed a high amount of SWCNTs were internalized by cells. Confocal Raman imaging showed that most of the internalized SWCNTs were distributed in the perinuclear region. The results indicated that SWCNTs could be internalized by chondrocytes when SWCNTs were incorporated in the three-dimensional biomimetic collagen hydrogels.

Keywords: Single-Walled Carbon Nanotubes, Collagen Hydrogles, Bovine Articular Chondrocytes, Cellular Uptake, Intracellular Distribution.

1. INTRODUCTION

One of the most important characteristics of carbon nanotubes (CNTs) is that they can effectively cross biological barriers[1,2] and therefore can be used as targeted and multi-delivery vehicles for diagnostically and therapeutically active molecules.[3,4] Meanwhile, their inherent optical, electrical and thermal properties make CNTs attractive candidates for biological imaging, detection and therapy.[3,5,6] Nevertheless, the main intrinsic drawback of CNTs is their extremely poor dispersibility in most of the common solvents due to their super-hydrophobicity and significant van der Waal's forces.[7,8] These features are associated with cytotoxicity and other negative cellular effects,[9] whereas well dispersed CNTs have no apparent cytotoxicity.[10,11] Furthermore, the success of many CNTs-based applications largely depends on how many CNTs can be internalized by cells. An efficient cellular uptake can greatly facilitate such applications in biomedicine and biotechnology. Two major pathways for the cellular uptake of CNTs have been confirmed. One describes CNTs as nanoneedles that can penetrate cell membranes in an energy-independent manner[12,13] and another one suggests that cellular uptake of CNTs performed via the clathrin-mediated endocytosis[14,15] which is an energy-dependent manner. In addition, adsorption of some biocompatible molecules, such as BSA, DNA and peptides, on the surfaces of CNTs may induce and facilitate receptor-mediated endocytosis of CNTs.[16–18] However, most of the cellular uptake studies *in vitro* have been performed with a two-dimensional (2D) cell culture system. 2D cell culture conditions are different from the microenvironment surrounding cells *in vivo*. As a result, the response of cells to CNTs, including cellular uptake, can not completely reflect the virtual behavior in their native tissue. One potential strategy to overcome the problem is to culture cells with a three-dimensional (3D) culture system which can mimic

*Author to whom correspondence should be addressed.

the natural extracellular matrix (ECM) and provide a functional support for cell growth, proliferation, differentiation and metabolism.[19–21]

Hydrogels with their beneficial characteristics, such as high water content, controllable biodegradability and excellent mass transfer properties, have been extensively explored for applications in 3D cell culture and regenerative medicine.[22,23] Compared with other materials, collagen-based hydrogles are of particular interest because collagen is the most abundant protein in mammals, shows regular helical structure, excellent biocompatibility and moderate immunogenicity.[24] Furthermore, collagen fibrils can physically assemble with each other to form a collagen network (fibrillogenesis) in aqueous solution. The resulting collagen hydrogels show a highly porous structure and can offer a three-dimensional biomimetic microenviroment for cells.

In this study, collagen hydrogels were used to investigate cellular uptake of single-walled carbon nanotubes (SWCNTs) in a biomimetic 3D culture system. SWCNTs were modified by coating their surfaces with collagen and incorporated in collagen hydrogels to form the composite hydrogels. Bovine articular chondrocytes were cultured in the composite hydrogels and cellular effects of SWCNTs were investigated.

2. MATERIALS AND METHODS

2.1. Preparation of SWCNTs/Collagen Composite Hydrogels

Prior to the preparation of SWCNTs/collagen composite hydrogels, SWCNTs (purity $>90\%$, 0.7–1.3 nm in diameter, Sigma-Aldrich, USA) were coated with collagen (collagen-coated SWCNTs) using a simple non-covalent approach to improve the dispersibility in water as reported before.[25] Briefly, 1,000 μg SWCNTs were sterilized using ultraviolet light for 2 hours and put in 10 mL 0.1 wt% collagen solution that was prepared by diluting 1.0 wt% collagen aqueous solution (type I collagen in $pH = 3.0$ acetic acid aqueous solution, Nippon Meat Packers Inc., Japan) with $pH = 3.0$ acetic acid aqueous solution. The mixture was sonicated (135 W, Bransonic, Japan) in an ice bath for 2 hours. The dispersion solution was then centrifuged (Tomy MX-301, Japan) at 20,000 g for 30 minutes to remove aggregated and bundled SWCNTs. To prepare SWCNTs/collagen composite hydrogels, the supernatant was mixed with 1.0 wt% collagen aqueous solution at a designated ratio. The SWCNTs-containing collagen solution was mixed with 10 times concentrated DMEM (Dulbecco's modified Eagle's medium, Sigma, USA) and HEPES buffer solution (50 mM NaOH, 260 mM $NaHCO_3$ and 200 mM HEPES) at a volume ratio of 8:1:1. All the solutions were pre-cooled at 4 °C before mixing and all the operations were performed in an ice bath. The mixtures were placed at 37 °C for gelation to form SWCNTs/collagen composite hydrogels.

2.2. Characterization of SWCNTs/Collagen Composite Hydrogels

The microstructures of the freeze-dried SWCNTs/collagen hydrogels were observed and imaged with a JSM-5610 scanning electron microscope (SEM, JEOL, Japan). Briefly, hydrogel samples were frozen at −80 °C and freeze-dried for 24 hours in a freeze-dryer under a vacuum of 5 Pa (VirTis AdVantage Benchtop Freeze Dryer, SP Industries Inc.). The freeze-dried specimens were plunged into liquid nitrogen and cut using a cold scalpel. The cross sections were then coated with platinum and observed by SEM at 10 kV accelerating voltage.

2.3. Cell Culture in Composite Hydrogels

Bovine articular chondrocytes (BACs) were isolated from the articular cartilage derived from a 9-week old female calf. Freshly isolated chondrocytes were defined as P0. BACs that were subcultured twice (*P*2) were used in this study. The BACs were cultured in 75 cm^2 tissue culture flasks (BD Falcon, USA) at 37 °C in humidified air containing 5% CO_2. The culture medium was DMEM supplemented with 10% fetal bovine serum, 4,500 mg/L glucose, 4 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 1 mM sodium pyruvate and 50 μg/mL ascorbic acid (serum DMEM). *P*2 cells were seeded into the composite hydrogels during hydrogel preparation. *P*2 cells were suspended in 10 times concentrated serum DMEM to prepare cell suspension solution at a density of 1.0×10^8 cells/cm^3. The SWCNTs-containing collagen solution, cell suspension solution and HEPES buffer solution were mixed at a volume ratio of 8:1:1. The cells-containing mixture solution was placed in 12-well plates and incubated at 37 °C in humidified air containing 5% CO_2 for 4 hours for formation of hydrogels. After hydrogel formation, 1.5 ml serum DMEM was added in each well. Cells cultured in SWCNTs-free collagen hydrogels, which was used as a control, were prepared by the same method as described above by using collagen solution without SWCNTs. Cell culture medium was changed every 3 days.

2.4. Cell Viability Assay

The proliferation of cells was evaluated by WST-1 assay. Firstly, the cell culture medium was replaced with 550 μL of WST-1 solution (Roche, Germany, 50 μL of WST-1 stock solution diluted with 500 μL of serum DMEM). After incubation for 3 hour, 100 μL of supernatant solution was collected and placed into a 96-well plate. The absorbance was measured at 440 nm using a plate reader (Benchmark Plus, USA).

2.5. Cellular Uptake of SWCNTs in the Composite Hydrogels

To determine the amount of SWCNTs internalized in the BACs, the cells were harvested by digesting the hydrogels

with type I collagenase/trypsin-EDTA solution and washed with warm PBS for 3 times. Then the washed cells were ruptured via a papain (Sigma, USA) buffer solution and the amount of SWCNTs in the buffer solution was measured by using UV-vis-NIR spectroscopy. The value was considered to be the amount of SWCNTs internalized in cells. Before being ruptured, the cells were counted with a cytometer.

2.6. Analysis of Intracellular Distribution of SWCNTs

An inverted confocal Raman microscope (AMANplus, Nanophoton, Japan) with a 532 nm laser (500 mW) and phase objectives (Nikon Microsystems, Japan) was used for both Raman and phase-contrast imaging. Briefly, the cells cultured in the hydrogels with or without SWCNTs for 7 days were harvested and re-cultured in a 4-chamber culture slide. And then the cells were fixed with 4% paraformaldehyde solution to prevent morphological and chemical changes during image acquisition. After that, the samples were washed with PBS and the slides were mounted and sealed using nail lacquer. A phase-contrast image of the cells was focused, revealing cellular morphologies including the nuclei and extensions. A horizontal confocal Raman image (x–y imaging mode) and an entire vertical section image (x–z imaging mode) were acquired to investigate the distribution of SWCNTs in cells. The confocal Raman spectra from 779.7 to 1970.0 cm^{-1} were collected and the G-band, indicative of SWCNTs, was used to map the distribution of SWCNTs. Control of imaging parameters, processing and data analysis were performed in a Raman Data Viewer software (Nanophoton, Japan).

2.7. Statistical Analysis

All data were reported as mean ± standard deviation (SD). One-way analysis of variance was performed to reveal statistical differences followed by Tukey's post hoc test for pairwise comparison. All statistical analyses were executed using Kyplot 2.0 beta 15.

3. RESULTS AND DISCUSSION

3.1. SWCNTs/Collagen Composite Hydrogels

Figure 1(a) shows the schematic illustration of the preparation of SWCNTs/collagen composite hydrogels for 3D culture of BACs. To improve their dispersibility in water, SWCNTs were coated with collagen at first. The collagen-coated SWCNTs retained the inherent properties of SWCNTs and the suspension solution was stable for months.[25] Most of the collagen-coated SWCNTs were individually dispersed in the suspension solution. SWCNTs/collagen composite hydrogels were prepared by adding the collagen-coated SWCNTs in collagen aqueous solution and gelation at neutral pH and 37 °C. The hydrogels were sticky and became static even when vessels were

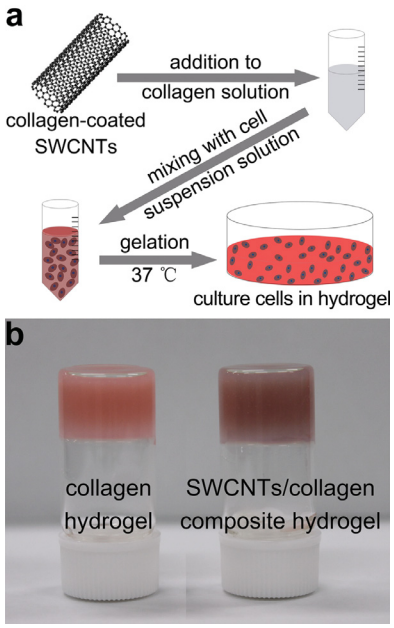

Figure 1. (a) Schematic illustration of the preparation of SWCNTs/collagen composite hydrogels for 3D culture of BACs. (b) Photographs of collagen hydrogel and SWCNTs/collagen composite hydrogel.

put upside down (Fig. 1(b)). The pink color was due to the color of the cell culture medium. The SWCNTs/collagen composite hydrogels were slightly black because of the incorporation of SWCNTs. The microstructure of collagen hydrogels and SWCNTs/collagen composite hydrogels

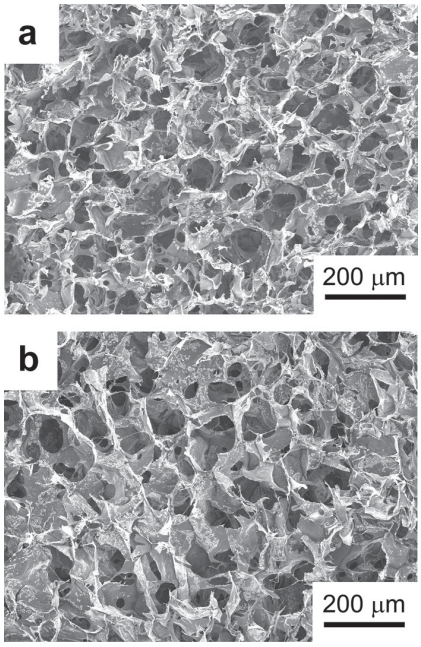

Figure 2. SEM images of freeze-dried (a) collagen hydrogel and (b) SWCNTs/collagen composite hydrogel.

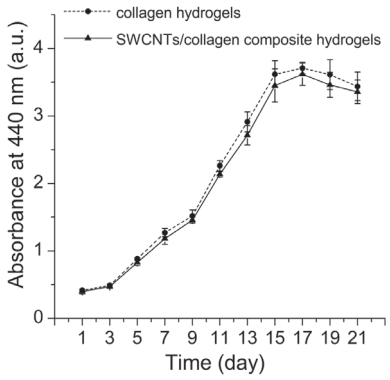

Figure 3. Proliferation curve of BACs during culture in the collagen hydrogels and SWCNTs/collagen composite hydrogels measured by a WST-1 assay.

were observed by SEM after freeze-drying. The collagen hydrogels and SWCNTs/collagen composite hydrogels showed the same microstructures (Fig. 2). There were many micropores in both hydrogels. The size of the micropores was around 100 μm. The micropore structure in the hydrogels should support cell movement and benefit diffusion of nutrients and metabolic substances.

3.2. Cell Viability in Hydrogels

The effect of SWCNTs on cell viability has been investigated *in vitro* by culturing cells with SWCNTs-containing culture medium. However, the published data are fiercely debated and extremely inconsistent.[9–11, 26] Figure 3 shows the results of WST-1 assays on cell viability cultured in collagen hydrogels and SWCNTs/collagen composite hydrogels over time. There was no significant difference between the two hydrogels, suggesting that the SWCNTs in the hydrogels did not affect cell viability. This result should be attributed to the good dispersibility of the modified SWCNTs and the bioactive collagen on the surface of nanotubes.[25]

3.3. Cellular Uptake of SWCNTs in Composite Hydrogels

There are many reports on the cellular uptake of SWCNTs. The effects of size, shape and surface functionalization of SWCNTs on internalization have been reported.[17, 27, 28] The results suggest that all of these parameters affect cellular uptake and surface adsorption of some biocompatible molecules, such as BSA, DNA and peptides, can be an important factor to affect cellular uptake of SWCNTs.[16, 17]

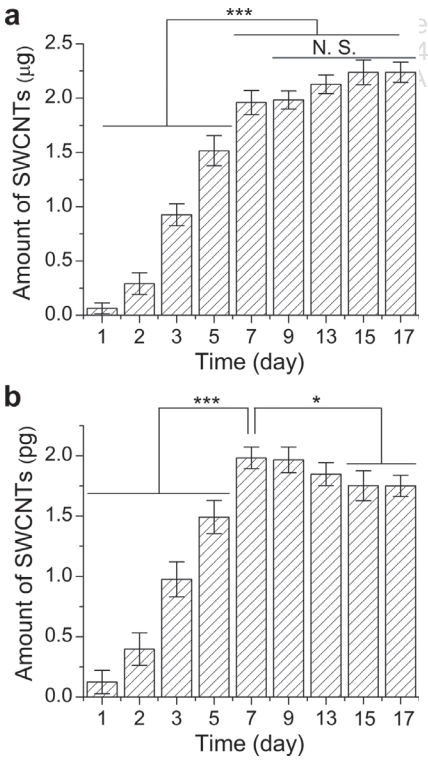

Figure 4. (a) Quantification of cellular uptake of SWCNTs in a BAC population after culture for 1 to 17 days. (b) Average amount of SWCNTs internalized in one cell on average after culture for 1 to 17 days. (N.S. indicates no significant difference, $^{*}p < 0.05$, $^{***}p < 0.001$).

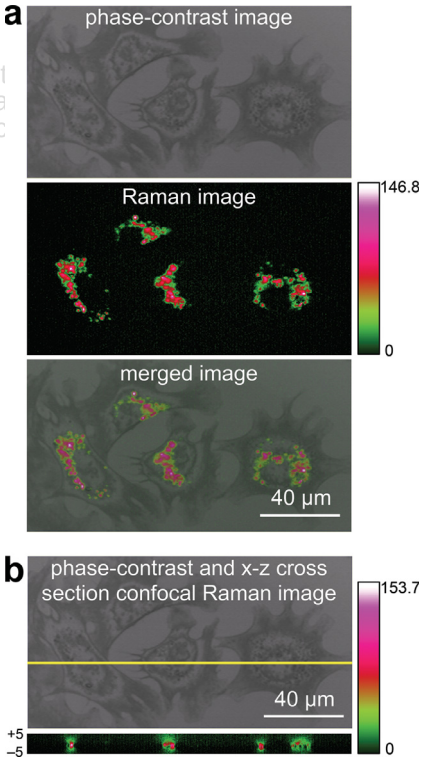

Figure 5. (a) Phase-contrast, Raman and merged micrographs and (b) *x–z* cross section confocal Raman image of a few cells after being cultured in SWCNTs/collagen composite hydrogels for 7 days. *x–z* cross section scanning was performed along the yellow line in the phase-contrast micrograph. The color-coded scale bar represents the *G*-band intensity of SWCNTs.

In this study, SWCNTs were detected in the cells when the cells were cultured in SWCNTs/collagen composite hydrogels. The amount of SWCNTs in the entire cell population increased with culture time until day 7 and remained unchanged since then (Fig. 4(a)). By dividing the total amount of internalized SWCNTs by cell number, the amount of SWCNTs internalized per cell was calculated (Fig. 4(b)). The uptake amount of SWCNTs per cell increased until day 7 and decreased slightly since day 7. Significant decrease was detected after culture for 15 and 17 days. At day 7, the amount of SWCNTs per cell was 1.98 ± 0.09 pg. In other words, up to $7.01 \pm 0.32 \times 10^6$ SWCNTs (molecular mass $\approx$ 170 kDa for length $\approx$ 235 nm, diameter $\approx$ 0.9 nm) were internalized by one cell on average. The high internalization amount of SWCNTs should benefit their applications for delivery of therapeutic and diagnostic biomolecules.

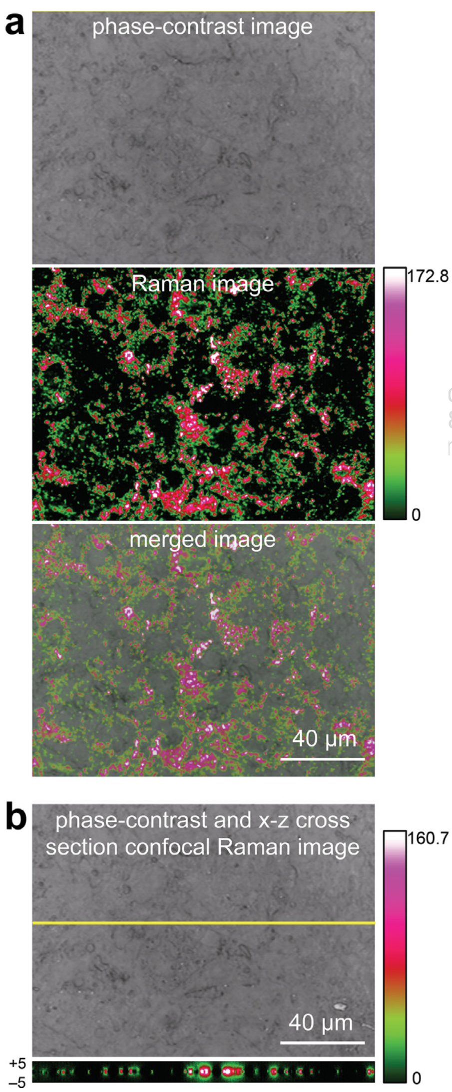

Figure 6. (a) Phase-contrast, Raman and merged micrographs and (b) *x–z* cross section confocal Raman image of a BAC population after being cultured in SWCNTs/collagen composite hydrogels for 7 days. *x–z* cross section scanning was performed along the yellow line in the phase-contrast micrograph. The color-coded scale bar represents the *G*-band intensity of SWCNTs.

3.4. Intracellular Distribution of Internalized SWCNTs

Raman spectroscopy as a label-free technique is a useful tool to visualize the SWCNTs in living cells.[16,25,29] Confocal Raman imaging was performed to investigate intracellular distribution of SWCNTs. Figure 5 shows the phase-contrast and confocal Raman images of cells using different scanning modes. The phase-contrast image clearly revealed the cellular morphology of BACs, including the cell shape, nucleus position and cell extension. In *x–y* scanning mode, the confocal Raman image showed that SWCNTs were primarily located around the nucleus (Fig. 5(a)). The result was consistent with previous reports[16,29,30] and supported by the *x–z* plane scanning confocal Raman imaging performed across the center of nucleus (Fig. 5(b)). By imaging a cell population (Figs. 6(a) and (b)), cellular uptake was confirmed within the population of cells and the intensity of SWCNTs was most prevalent in the perinuclear region. The results indicated that SWCNTs could be internalized in cells when hydrogels were used for 3D cell culture. The internalized SWCNTs were predominantly distributed around the nuclei of cells. The results suggest SWCNTs/collagen composite hydrogels may be useful for the delivery of proteins, drugs and genes for biomedical applications.

4. CONCLUSIONS

Cellular uptake of SWCNTs in a 3D culture condition was investigated by culturing chondrocytes in SWCNTs/collagen composite hydrogels. The SWCNTs/collagen hydrogels showed no negative effects on the viability of chondrocytes. Uptake of SWCNTs by chondrocytes during 3D culture was confirmed by using UV-vis-NIR spectroscopy and $7.01 \pm 0.32 \times 10^6$ SWCNTs were internalized in one cell on average. The internalized SWCNTs were prevalently accumulated in the perinuclear region. The results indicated that SWCNTs could be internalized by cells when being cultured in the 3D biomimetic collagen hydrogels.

Acknowledgments: This work was supported partially by the World Premier International Research Center Initiative (WPI) and partially by "Nanotechnology Network Project" of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References and Notes

1. S. Pogodin and V. A. Baulin, *ACS Nano* 4, 5293 (**2010**).
2. S. Höfinger, M. Melle-Franco, T. Gallo, A. Cantelli, M. Calvaresi, J. A. N. F. Gomes, and F. Zerbetto, *Biomaterials* 32, 7079 (**2011**).

3. Z. Liu, J. T. Robinson, S. M. Tabakman, K. Yang, and H. Dai, *Mater. Today* 14, 316 **(2011)**.
4. J. Ren, S. Shen, D. Wang, Z. Xi, L. Guo, Z. Pang, Y. Qian, X. Sun, and X. Jiang, *Biomaterials* 33, 3324 **(2012)**.
5. A. E. Porter, M. Gass, K. Muller, J. N. Skepper, P. A. Midgley, and M. Welland, *Nat. Nanotech.* 2, 713 **(2007)**.
6. K. S. Yun, J. S. Yun, J. C. Kim, J. Min, T. J. Park, J. K. Ahn, and D. H. Kim, *J. Nanosci. Nanotechnol.* 12, 377 **(2012)**.
7. K. E. Geckeler and T. Premkumar, *Nanoscale Res. Lett.* 6, 136 **(2011)**.
8. R. Bandyopadhyaya, E. Nativ-Roth, O. Regev, and R. Yerushalmi-Rozen, *Nano Lett.* 2, 25 **(2002)**.
9. D. Liu, C. Yi, D. Zhang, J. Zhang, and M. Yang, *ACS Nano* 4, 2185 **(2010)**.
10. N. W. S. Kam, Z. Liu, and H. Dai, *Angew. Chem. Int. Ed.* 45, 577 **(2006)**.
11. N. Wong, S. Kam, T. C. Jessop, P. A. Wender, and H. Dai, *J. Am. Chem. Soc.* 126, 6850 **(2004)**.
12. D. Pantarotto, J.-P. Briand, M. Prato, and A. Bianco, *Chem. Commun.* 1, 16 **(2004)**.
13. Q. Lu, J. M. Moore, G. Huang, A. S. Mount, A. M. Rao, L. L. Larcom, and P. C. Ke, *Nano Lett.* 4, 2473 **(2004)**.
14. G. Hong, J. Z. Wu, J. T. Robinson, H. Wang, B. Zhang, and H. Dai, *Nat. Commun.* 3, 1 **(2012)**.
15. K. T. Al-Jamal, H. Nerl, K. H. Müller, H. Ali-Boucetta, S. Li, P. D. Haynes, J. R. Jinschek, M. Prato, A. Bianco, K. Kostarelos, and A. E. Porter, *Nanoscale* 3, 2627 **(2011)**.
16. B. D. Holt, K. N. Dahl, and M. F. Islam, *Small* 7, 2348 **(2011)**.
17. M. L. Becker, J. A. Fagan, N. D. Gallant, B. J. Bauer, V. Bajpai, E. K. Hobbie, S. H. Lacerda, K. B. Migler, and J. P. Jakupciak, *Adv. Mater.* 19, 939 **(2007)**.
18. D. A. Tsyboulski, E. L. Bakota, L. S. Witus, J. D. R. Rocha, J. D. Hartgerink, and R. B. Weisman, *J. Am. Chem. Soc.* 130, 17134 **(2008)**.
19. L. Haines-Butterick, K. Rajagopal, M. Branco, D. Salick, R. Rughani, M. Pilarz, M. S. Lamm, D. J. Pochan, and J. P. Schneider, *Proc. Natl. Acad. Sci. USA* 104, 7791 **(2007)**.
20. J. Kisiday, M. Jin, and B. Kurz, *Proc. Natl. Acad. Sci. USA* 99, 9996 **(2002)**.
21. S. Zhang, *Nat. Biotechnol.* 21, 1171 **(2003)**.
22. K. Y. Lee and D. J. Mooney, *Chem. Rev.* 101, 1869 **(2001)**.
23. G. D. Nicodemus and S. J. Bryant, *Tissue Eng. PT B-Rev.* 14, 149 **(2008)**.
24. F. R. Tian, D. X. Cui, H. Schwarz, G. G. Estrada, and H. Kobayashi, *Toxicol. In Vitro* 20, 1202 **(2006)**.
25. H. Mao, N. Kawazoe, and G. Chen, *Biomaterials* 34, 2472 **(2013)**.
26. Y. Zhang, Y. Xu, Z. Li, T. Chen, S. M. Lantz, P. C. Howard, M. G. Paule, W. Slikker, F. Watanabe, T. Mustafa, A. S. Biris, and S. F. Ali, *ACS Nano* 5, 7020 **(2011)**.
27. G. Romero, I. Estrela-Lopis, E. Rojas, I. Llarena, E. Donath, and S. E. Moya, *J. Nanosci. Nanotechnol.* 12, 4836 **(2012)**.
28. H. Jin, D. A. Heller, R. Sharma, and M. S. Strano, *ACS Nano* 3, 149 **(2009)**.
29. A. E. Porter, M. Gass, J. S. Bendall, K. Muller, A. Goode, J. N. Skepper, P. A. Midgley, and M. Welland, *ACS Nano* 3, 1485 **(2009)**.
30. L. Lacerda, G. Pastorin, D. Gathercole, J. Buddle, M. Prato, A. Bianco, and K. Kostarelos, *Adv. Mater.* 19, 1480 **(2007)**.

Received: 30 December 2012. Accepted: 8 February 2013.