Poly(2-(methacryloyloxy) ethyl phosphorylcholine)-Functionalized Multi-walled Carbon Nanotubes: Preparation, Characterization, Solubility, and Effects on Blood Coagulation

Leize Zhu, Qiao Jin, Jianping Xu, Jian Ji, Jiacong Shen

Department of Polymer Science, Key Laboratory of Macromolecule Synthesis and Functionalization of Minister of Education, Zhejiang University, Hangzhou 310027, China

Received 19 June 2008; accepted 21 November 2008
DOI 10.1002/app.29750
Published online 19 March 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Water-soluble multi-walled carbon nanotubes (MWNTs) were prepared via surface-initiated atom transfer radical polymerization (ATRP) of 2-(methacryloyloxy) ethyl phosphorylcholine (MPC) from carbon nanotubes (CNTs). The success of the surface functionalization of MWNTs with poly(2-(methacryloyloxy) ethyl phosphorylcholine) (pMPC) was ascertained using fourier transform infrared spectrophotometry (FTIR), thermogravimetric analysis (TGA), hydrogen nuclear magnetic resonance (^{1}H-NMR), and transmission electron microscopy (TEM). Different from the results of the previous work, in our work, we demonstrate that the amount of pMPC on CNTs can be easily regulated by ATRP approach. In addition, from TGA results, a linear relationship between the weight loss fraction of MWNT-pMPC and the weight of MPC fed and as high as 48.1% weight loss of MWNT-pMPC (MWNTs grafted by pMPC) are observed. Through TEM, the core-shell structure of MWNT-pMPC is clearly observed, which is also different from the previous report. The pMPC-modified MWNTs are highly soluble, which can also resist pH and saline concentration changes and remain stable in physiological environment. PMPC-modified MWNT does not significantly affect the blood coagulation as demonstrated in plasma recalcification time (PRT) test. These highly soluble MWNTs are expected to enable their wide use in biomedical areas. J Appl Polym Sci 113: 351–357, 2009

Key words: atom transfer radical polymerization (ATRP); phospholipids; modification; biocompatibility; carbon nanotubes (CNTs)

INTRODUCTION

The past decades have witnessed a tremendous growth in the exploration of carbon nanotubes for applications in biosensors,[1] bioimaging,[2] tissue engineering,[3] drug delivery systems,[4] and membrane for bioseparation.[5] However, the advancement of biological and biomedical application of CNTs has been impeded because of the highly hydrophobic nature of CNTs.[6] One route to disperse nanotubes involves chemical functionalization of polymer, either through "graft to"[7] or "graft from"[8] approach. The use of "graft from" strategy is preferred over the "graft to" method because a large quantity of polymer can be anchored onto the MWNTs due to the less steric hindrance of the attached macromolecules. Among "graft from" strategies, surface-initiated atom transfer radical polymerization is a powerful technique in growing dense polymer layer on CNTs.[9]

On solving the problem of the aqueous dispersion of CNTs, blood compatibility should be concerned as well.[10] The lack of blood compatibility that is responsible for the adsorption of plasma proteins, platelet adhesion, and activation often leads to the clot formation[11] and the failure of CNTs-based devices *in vivo*. So, the far-sighted choice of the polymer to disperse CNTs is essential for their biomedical potential applications. However, until recently, only a few methods have been reported to impart blood compatible surface to the nanomaterials, especially the CNTs. So-called "PEGylated"[12] or "heparinized"[13] are of particular interests among these strategies.

Inspired by the phospholipid head groups located on the surface of cell membranes, several groups have developed phosphorylcholine-based materials. For example, the medical devices, surface modified with phosphorylcholine groups has excellent blood

Correspondence to: J. Ji (jijian@zju.edu.cn).
Contract grant sponsor: Natural Science Foundation of China; contract grant numbers: NSFC-20774082, 50830106.
Contract grant sponsor: Program for New Century Excellent Talents in University; contract grant number: NCET-05-0527.
Contract grant sponsor: National High Technology Research and Development Program of China; contract grant numbers: 2006AA03Z329, 2006AA032444.
Contract grant sponsor: Science and Technology Projects of Zhejiang Province; contract grant number: 2007C24008.

compatibility.[14] pMPC-based hydrogels,[15] micelles,[16] and vesicles[17] have been developed as controlled drug delivery systems. Recently, Chen and Armes[18] have employed pMPC to modify the surface of inorganic nanoparticles to combine the high biocompatibility of pMPC with intrinsic properties of nanomaterials. Hence, we hypothesize here that pMPC functionalized CNTs will not only exhibit highly aqueous solubility in physiological environment but also improve blood compatibility significantly.

Narain et al.[19] recently reported to have developed single-walled carbon nanotubes (SWNTs)—pMPC composites via ATRP. However, the high cost of SWNTs (ca. \$500 g^{-1} for kilogram-scale production) severely restricts its commercialization for all but very specialized applications. This problem can be mitigated using MWNTs.[20] In addition, there are still some problems that remain unsolved by Narain et al. First, whether the thickness of the polymer layer on the carbon nanotubes can be easily regulated through ATRP approach? Second, whether MWNT-pMPC can be dispersed in complex physiological solution? Third, whether MWNT-pMPC significantly affect the blood coagulation? These questions are crucial for surface engineering the MWNT with pMPC and extending the same to biomedical applications. Herein, we report the preparation of water-soluble MWNTs via surface-initiated atom transfer radical polymerization of MPC in mixed solvent with deactivator. The surface engineering processes were followed by with X-ray photoelectron spectroscopy (XPS), FTIR, ^{1}H-NMR, and TGA. Structural and biological characterizations of the pMPC-modified MWNTs were undertaken by TEM and PRT test, respectively.

EXPERIMENTAL

Materials

MWNT used in this study was purchased from Shenzhen Nanotech Port Co. MPC was synthesized and purified according to the reported procedures,[21] CuBr was purified by washing with glacial acetic acid, followed by absolute ethanol and ethyl ether, and then dried under vacuum. 2, 2′-bipyridyl (bpy), ethyl 2-bromoisobutyrate and $CuBr_2$ were purchased from Aldrich. Ethanol and tetrahydrofuran (THF) were obtained from domestic market and used without further purification.

Instrumentations

^{1}H-NMR spectra were measured with a Bruker Advance 400/500 DMX NMR spectrometer with D_2O as solvent. TEM analysis was conducted on a JEM-1230 (Jeol, Japan) electron microscope at 80 kV. TGA were carried out on a Perkin-Elmer Pyris thermogravimetric analyzer TGA 6 from 50 to 700°C at a heating rate of 20°C/min in the nitrogen flow (40 mL/min). FTIR spectra were obtained on a Bruker VECTOR 22 Spectrometer using KBr pellets. Filtration was done through a 0.22 μ Millipore polytetrafluroethylene (PTFE) membrane. UV/Vis spectra were recorded on a UV/Vis spectrophotometer (Shimadzu UV-2550). XPS characterization was conducted on a ESCALAB MARK spectrometer by using Mg Kα as exciting radiation.

Synthesis of MWNT-COOH, MWNT-OH, and MWNT-Br

Carbon nanotubes with functional groups of carboxyl (MWNT-COOH), hydroxyl (MWNT-OH), and MWNTs-based macroinitiator (MWNT-Br) were synthesized according to the procedures reported by Gao and coworkers[9,22]

Synthesis of MWNT-pMPC

In a typical polymerization reaction (MWNT-pMPC-b in Table I), 75.0 mg (0.013 mmol Br) MWNT-Br,1.0 mL pure water and 0.5 mL ethanol were placed in a flask, and then, the flask was evacuated and filled thrice with Ar. After that, 7.5 mg (0.052 mmol) of CuBr, 0.8 mg (0.0052 mmol) of $CuBr_2$, and 17.9 mg (0.11 mmol) of bpy were added to the stirred mixture under Argon. 134.2 mg (0.45 mmol) of MPC was added as solid to the flask at last. 24 h later, polymerization was terminated on exposure to air. To ensure that no ungrafted polymer or free reagents were mixed with the product, the filtered solid was dispersed in deionized water for 72 h, then, the surfactant was filtered and washed thrice with

TABLE I
Reaction Conditions and Results Initiated with MWNT-Br or Ethyl 2-Bromoisobutyrate

Samples	*R* (mol)	Sol. (mL)	*F*, wt (%)	*T* (nm)
MWNT-pMPC-a	18 : 1 : 4 : 0.4 : 8.8	1.0/0.5	20.9	<5
MWNT-pMPC-b	35 : 1 : 4 : 0.4 : 8.8	1.0/0.5	25.9	10–15
MWNT-pMPC-c	70 : 1 : 4 : 0.4 : 8.8	1.0/0.5	48.1	40–45
MWNT-pMPC-d	140 : 1 : 4 : 0.4 : 8.8	1.0/0.5	46.8	–
pMPC	70 : 1[g] : 1 : 0 : 2	2.0/1.0	73.4	–

R, MPC : initiating sites on MWNT-Br : Cu(I)Br : Cu(II)Br_2 : bpy(mol : mol : mol : mol).

Sol, solvent (vol./vol.) = water/ethanol (mL/mL).

F, the weight loss fraction of samples calculated from TGA.

a, b, c, d, MWNT-pMPC obtained by different feed ratios.

T, mean thickness of the grafted polymer calculated from TEM images.

[g] Pure pMPC was initiated with ethyl 2-bromoisobutyrate.

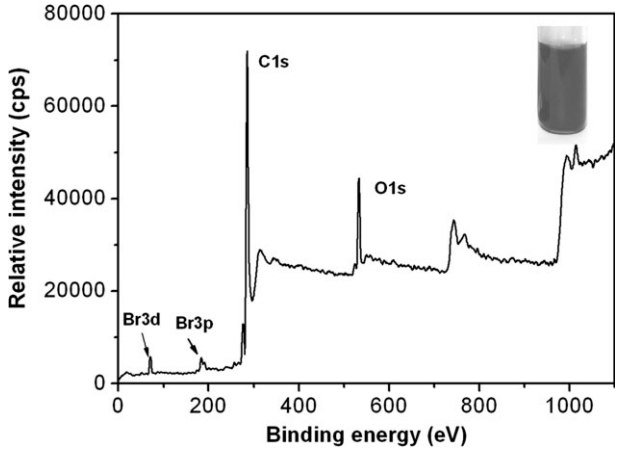


Figure 1 XPS spectrum of MWNT-Br. Insert: photo of MWNT-Br dispersed in chloroform.

deionized water. The resulting solid (MWNT-pMPC) was obtained by drying overnight under vacuum.

ATRP of MPC without MWNTS

The ATRP of MPC without MWNTs was carried out in a mixed solvent (water/ethanol 2.0 mL/1.0 mL) and initiated by ethyl 2-bromoisobutyrate: Typically, 10.1 mg (0.052 mmol) of ethyl 2-bromoisobutyrate, 2.0 mL pure water, and 1.0 mL ethanol were placed in a 5 mL flask and then the flask was evacuated and filled thrice with Ar. After that, 7.5 mg (0.052 mmol) of CuBr and 16.2 mg (0.10 mmol) of bpy were added to the stirred mixture under Argon. 1081.8 mg (3.64 mmol) of MPC was then added as solid to the flask. The flask was kept at room temperature for 24 h; Polymerization was terminated on exposure to air. The resulting mixture was precipitated into THF, then redissolved in water, and passed through a silica column to remove residual ATRP catalyst. After drying in a vacuum, pure pMPC was obtained. ($\overline{M_n}$ = 18,000 g/mol, $\overline{M_W}/\overline{M_n} = 1.19$ by GPC)

Plasma recalcification time

Plasma recalcification time test was performed according to the method used previously by our group with a slight modification.[23] An amount of 100 μL of MWNTs (MWNT-COOH, MWNT-OH, MWNT-pMPC-b, respectively) solution (1.0 mg/mL) and 100 μL of fresh human plasma obtained from Blood center of Zhejiang Province in which the Ca^{2+} was chelated using citrate were incubated in a siliconized vial for 5 min at 37°C. After that, 100 μL of previously warmed (37°C) calcium chloride solution (0.025*M*) was added into the tube, and the stop-watch started. At the same time, a small stainless steel hook was used to stir the recalcified plasma until the silky fibrin appeared. And this time was recorded as plasma recalcification time. Blank control experiments were conducted by adding 100 μL of 0.9% NaCl solution in place of MWNTs solution. Data were presented as a mean standard deviation of five different experiments.

RESULTS AND DISCUSSION

Preparation of MWNT-Br

Peaks at 70.0 eV and 184.0 eV corresponding to Br 3d and 3p electrons are clearly seen in XPS spectrum indicating the existence of Br on the CNTs (Fig. 1). In TGA measurements, no significant weight loss is observed from 50 to 700°C for pristine MWNTs [Fig. 2(Aa)]. While, MWNT-Br shows 13.6% weight loss below 450°C [Fig. 2(Ac)] and 11.0% weight loss for MWNT-OH [Fig. 2(Ab)], the additional 2.6% weight loss of MWNT-Br compared to MWNT-OH is attributable to the attachment of ATRP initiator (0.174 mmol Br per gram of MWNT-Br[22]). The corresponding FTIR

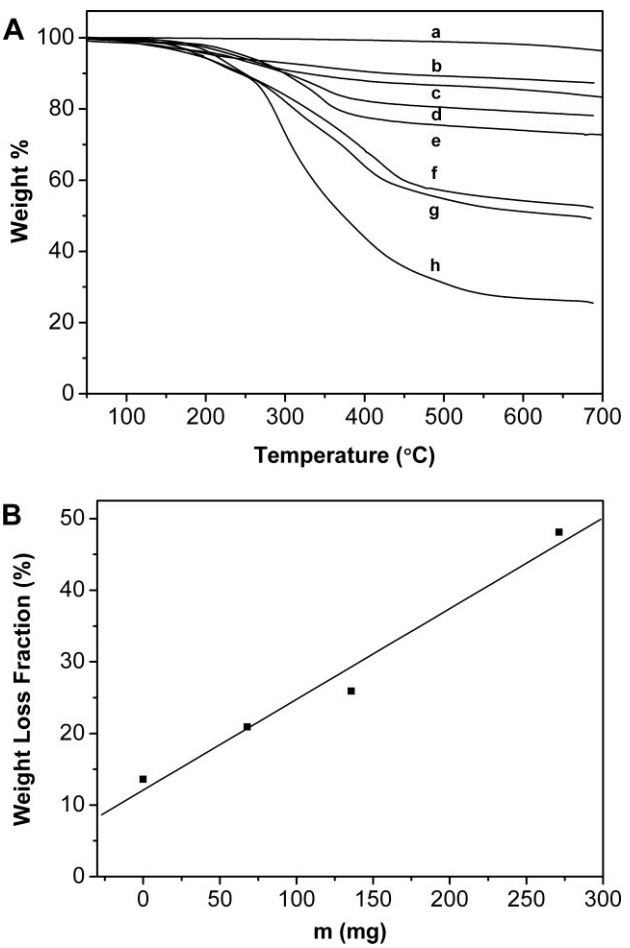


Figure 2 (A) TGA weight loss curves of pristine MWNTs (a), MWNT-OH (b), MWNT-Br (c), MWNT-pMPC-a (d), MWNT-pMPC-b (e), MWNT-pMPC-d (f), MWNT-pMPC-c (g), and pure pMPC (h). (B) The weight loss fraction of MWNT-pMPC as a function of the weight of MPC fed.

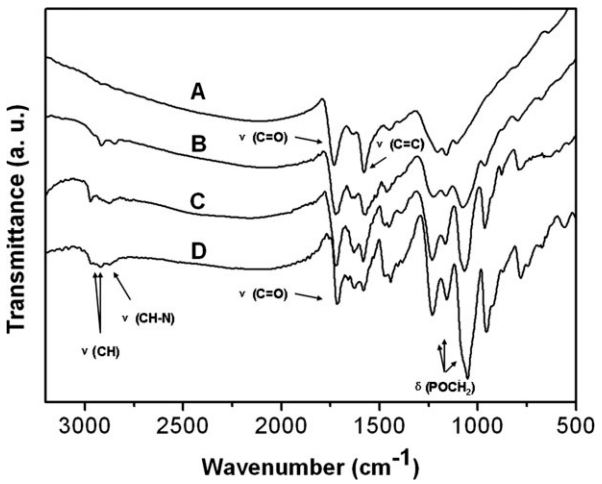


Figure 3 FTIR spectra of MWNT-Br (A), MWNT-pMPC-a (B), MWNT-pMPC-b (C), MWNT-pMPC-c (D).

spectrum of MWNT-Br is also shown in Figure 3. The resultant MWNT-Br can be well dispersed in chloroform (Fig. 1 insert).

Preparation of MWNT-pMPC

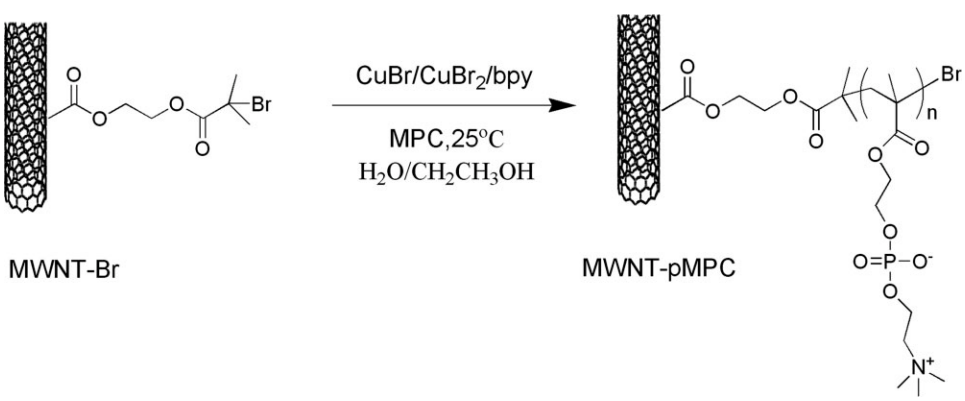
Recently, it has been demonstrated that MPC can be homopolymerized or copolymerized via ATRP facilely.[24] For the ATRP, the concentration of monomers and the solvents are important.[25] Thus a high concentration of monomers was employed, 1 : 2 mixture of ethanol/H_2O was used as the solvent system which allowed for adequate dispersion of the macroinitiators, and $CuBr_2$/CuBr/bpy as the catalyst/ligand[26] combination in this experiment. The reaction conditions and results are summarized in Table I. The synthesis of MWNT-pMPC is displayed in Scheme 1.

The chemical structure of the MWNT-pMPC is characterized by FTIR and ^{1}H-NMR. Absorptions at 1062 cm^{-1}, 1156 cm^{-1}, and 1230 cm^{-1} are due to —$POCH_2$— bend vibration, 2807 cm^{-1} is due to —CH_2—N— stretch vibration, and —CH_2— stretch vibration appeared at 2850 and 2930 cm^{-1}. An increase of the intensity of the absorption at 1062, 1156, 1230, 2807, 2850, and 2930 cm^{-1} of the resultant MWNT-pMPC with the increase of the feed ratio of MPC to MWNT-Br is also clearly observed (Fig. 3), as the amount of polymer grafted on MWNT increases by tuning the feed ratio. (MPC: initiating sites on MWNT-Br (mol/mol), the weight ratio of MWNT-pMPC to KBr is a constant in FTIR measurements).

The ^{1}H-NMR spectra of MWNT-pMPC and pMPC are the same [Fig. 4(A,B)]. The corresponding proton peaks of the grafted pMPC chains appear in the ^{1}H-NMR spectrum of MWNT-pMPC at δ 3.09 (—$N(CH_3)_3$), 3.52–3.54 (—CH_2N—), 4.03–4.05 (—$POCH_2CH_2N$—), 4.16–4.17 (—$COOCH_2$—), 4.26–4.28 (—CH_2CH_2OP—), respectively, [Fig. 4(B)]. The absence of the vinyl signals between δ 5.5 and 6.0 indicates that the unreacted monomers have been successfully removed through the purification procedures.

The weight loss fraction of MWNT-pMPC, which is corresponding to the quantity of pMPC on the MWNTs, is determined by TGA. Weight loss of as high as 48.1% is observed for MWNT-pMPC. From Figure 2(B), we observe that the weight loss fraction increases linearly with the increase of the weight of MPC fed. This again demonstrates that the amount of polymer grafted can be tuned by the feed ratio. However, no higher weight loss fraction of MWNT-pMPC is observed when the feed ratio further increases to 140 : 1 in this study.

The TEM images shown in Figure 5 further demonstrate the successful introduction of pMPC onto the MWNTs. With the increasing of the feed ratio, the polymer layer on the nanotubes become thicker (Table I, from thinner than 5 nm to ∼ 40–45 nm). Core-shell structure can be clearly seen from MWNT-pMPC-a to MWNT-pMPC-c. While in Narain et al.[19]



Scheme 1 Synthesis of pMPC functionalized MWNTs.

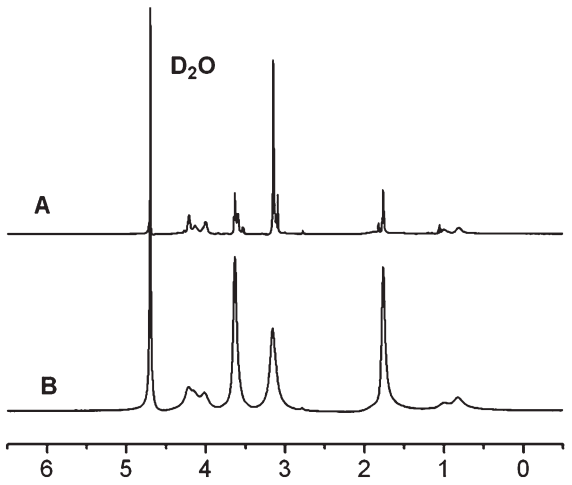


Figure 4 ^{1}H-NMR spectra of pristine pMPC (A), MWNT-pMPC (B) in D_2O.

results, a continuous polymer layer is formed among carbon nanotubes, as CNT-Br cannot be well-dispersed by water [Fig. 7 (Ab)]. In addition, from the image of MWNT-pMPC-c stained with phosphotungstic acid [Fig. 5(E)], we observed that one long CNT along with two short ones formed a "trimer" and the periphery of the pMPC grafted on MWNT can be clearly seen in this image.

Solubility

The UV/Vis spectra of MWNT-pMPC at different concentrations are shown in Figure 6. Because pMPC does not show absorption in the observed region, the absorption is exclusively caused by MWNTs. The dependence of absorbance at 600 nm on solution concentration apparently obeys Beer's law, indicating

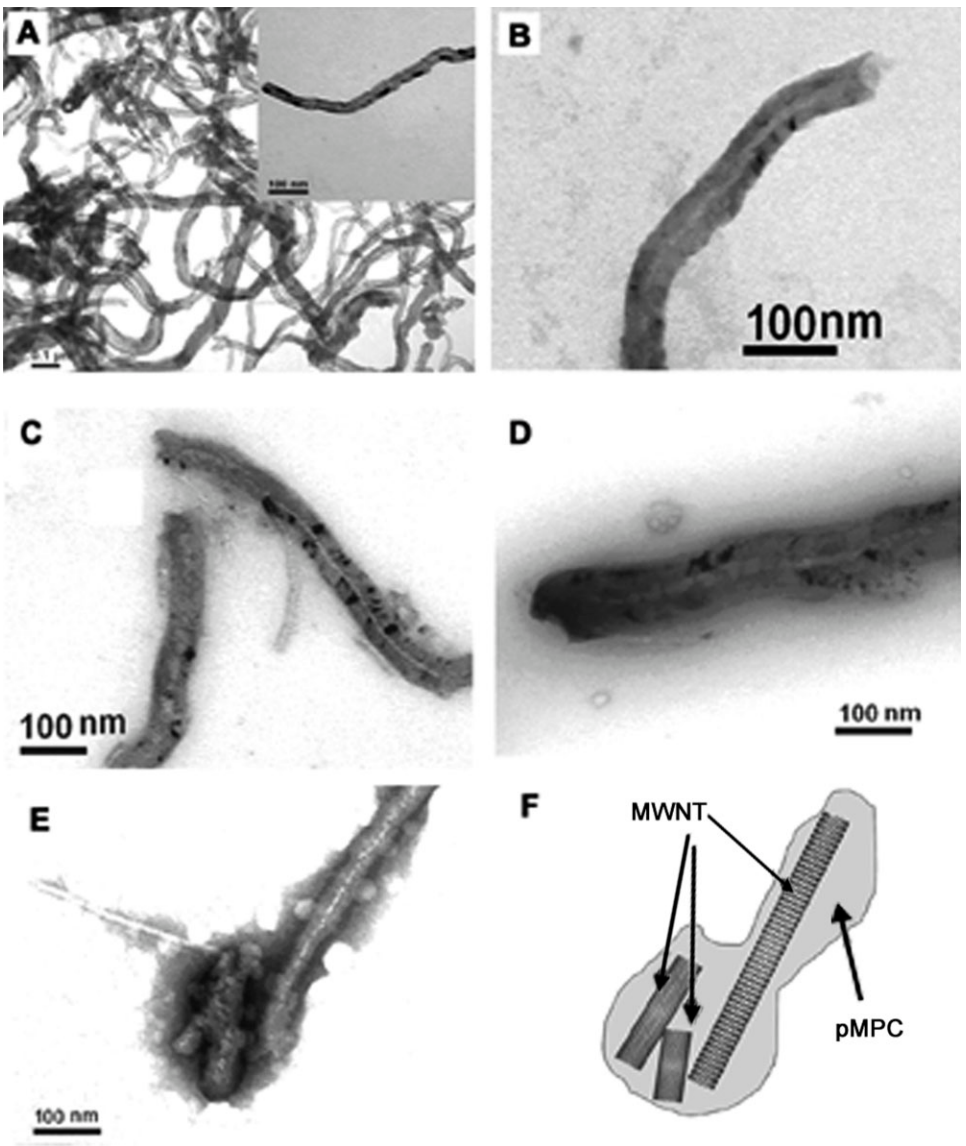


Figure 5 Representative TEM images of pristine MWNTs (A), MWNT-pMPC-a (B), MWNT-pMPC-b (C), MWNT-pMPC-c (D), and MWNT-pMPC-c treated with phosphotungstic acid (E), a cartoon (F) also shown to illustrate the "trimer".

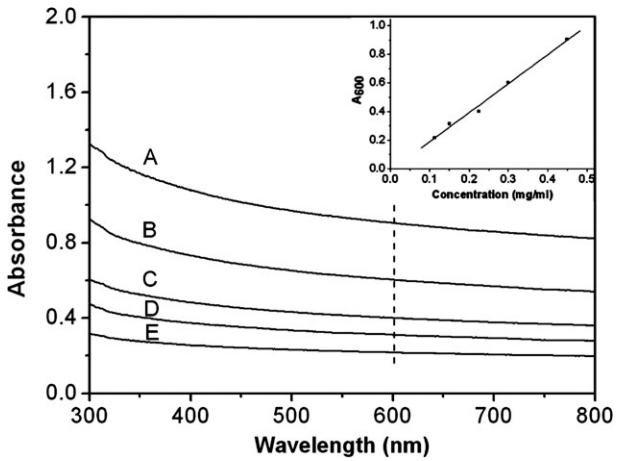


Figure 6 UV/Vis absorption spectra of MWNT-pMPC in aqueous solution at concentrations (mg/mL) of 0.113 (A), 0.150 (B), 0.225 (C), 0.300 (D), 0.450 (E). Insert: the absorbance at 600 nm of MWNT-pMPC as a function of the concentrations of MWNT-pMPC.

homogenous dispersion of the modified MWNTs is formed in solution.[27]

As can be seen from Figure 7(Aa) and Figure 7(Ab), pristine MWNTs and MWNT-Br cannot be dispersed in deionized water, and precipitate upon being added into bottles. The mechanical blending of MWNT-Br and pMPC does not result in stabilized MWNTs and the MWNTs precipitate from deionized water 3 days later. However, the covalent attachment of pMPC onto MWNTs affords well dispersed MWNTs in the deionized water and no precipitate is observed after 3 days. Figure 7(C) shows MWNT-pMPC dispersed in 10 m*M* phosphate buffer (PB) (pH = 1, 5, 7, 9, 13, respectively), the solubility in phosphate-buffered saline (0.1*M* PBS), and the fresh human plasma. Despite different pH of PB, MWNT-pMPC can be well dispersed and form transparent suspensions. After the MWNT-pMPC was added into physiological PBS solution and human plasma, MWNT-pMPC can be well dispersed too. These results indicate that MWNT-pMPC can resist pH and saline concentration changes and remain stable in physiological environment.

Blood compatibility

The PRT test was performed to determine the blood compatibility of the functionalized MWNTs. As pMPC is a known biocompatible polymer, the introduction of pMPC onto the MWNTs is thought to be able to increase the biocompatibility of MWNTs significantly. As can be seen from Figure 8, PRT obtained with the as-synthesized MWNT-COOH and MWNT-OH are shorter than that in the control experiment with saline buffer, indicating the interaction of MWNTs with the blood components. On the other hand, the PRT obtained with MWNT-pMPC is comparable to that for the saline control. This indicates that pMPC-modified MWNT does not

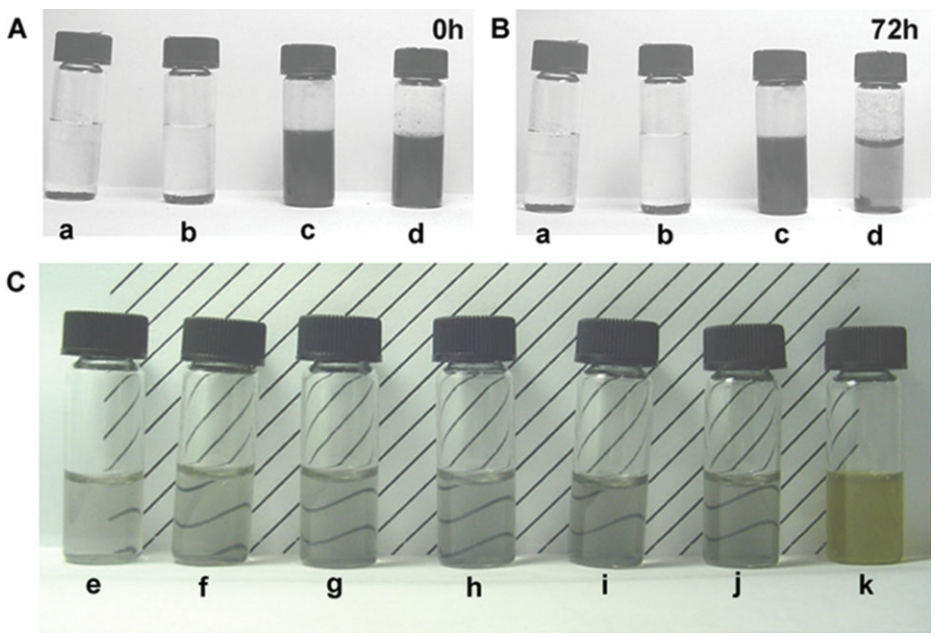


Figure 7 (A) Photographs of MWNTs (a), MWNT-Br (b), MWNT-pMPC (c), and MWNT-Br/pMPC mixture (d) in deionized water. All the samples are stirred for 2 min, and photographs were taken 5 min after putting bottles on Table. (B) After 72 h. (C) Photographs of 0.072 mg/mL MWNT-pMPC in pH = 1 (e), pH = 5 (f), pH = 7 (g), pH = 9 (h), pH = 13 (i) 10 m*M* PB solution, pH = 7.4 0.1*M* PBS (j) and the fresh human plasma (k). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.].

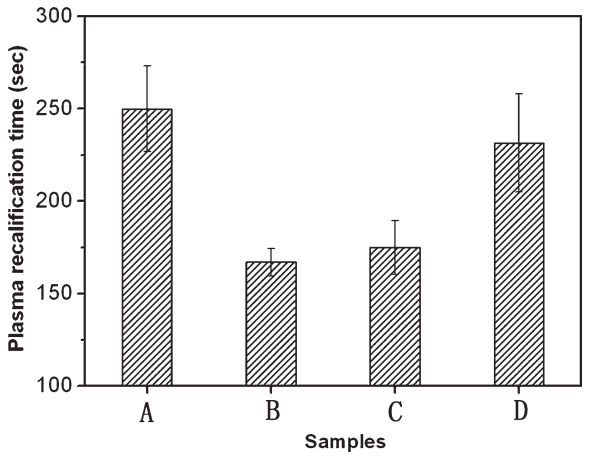


Figure 8 Data of PRT. The samples are 0.9% NaCl (A), MWNT-COOH (B), MWNT-OH (C), MWNT-pMPC-b (D), respectively. Data are presented as a mean standard deviation of five different experiments.

significantly affect the blood coagulation and may be more blood compatible than the unmodified MWNT.[13,28]

CONCLUSIONS

In summary, a bioinspired functionalized carbon nanotube is successfully synthesized via surface-initiated ATRP of MPC from MWNTs in mixed solvent with deactivator at room temperature. The pMPC content on CNT can be easily regulated by ATRP approach and as high as 48.1% weight loss of MWNT-pMPC is achieved. A linear relationship between the weight loss fraction of MWNT-pMPC and the weight of MPC fed is also observed. The pMPC-modified MWNTs exhibit highly aqueous solubility as determined by UV/Vis spectra, which can also resist pH and saline concentration changes and remain stable in physiological environment. pMPC-modified MWNT does not significantly affect the blood coagulation. These highly water-soluble MWNTs are expected to be used in aqueous coatings and composites for biomedical devices.

References

1. Lee, H.; Yoon, S. W.; Kim, E. J.; Park, J. Nano Lett 2007, 7, 778.
2. Bottini, M.; Cerignoli, F.; Dawson, M. I.; Magrini, A.; Rosato, N.; Mustelin, T. Biomacromolecules 2006, 7, 2259.
3. Harrison, B. S.; Atala, A. Biomaterials 2007, 28, 344.
4. Liu, Z.; Winters, M.; Holodniy, M.; Dai, H. J Angew Chem Int Ed 2007, 46 2023.
5. Srivastava, A.; Srivastava, O. N.; Talapatra, S.; Vajtai, R.; Ajayan, P. M. Nat Mater 2004, 3, 610.
6. Lin, Y.; Taylor, S.; Li, H. P.; Shiral Fernando, K. A.; Qu, L. W.; Wang, W. J Mater Chem 2004, 14, 527.
7. Chattopadhyay, J.; Cortez, F. D. J.; Chakraborty, S.; Slater, N. K. H.; Billups, W. E. Chem Mater 2006, 18, 5864.
8. Xu, G. Y.; Wu, W. T.; Wang, Y. S.; Pang, W. M.; Wang, P. H.; Zhu, Q. R. Nanotechnology 2006, 17, 2458.
9. Kong, H.; Gao, C.; Yan, D. Y. J Am Chem Soc 2004, 126, 412.
10. Lacerda, L.; Bianco, A.; Prato, M.; Kostarelos, K. Adv Drug Delivery Rev 2006, 58, 1460.
11. Ratner, B. D. J Biomater Sci Polym Ed 2000, 11, 1107.
12. Liu, Z.; Cai, W. B.; He, L. N.; Nakayama, N.; Chen, K.; Sun, X. M. Nat Nanotech 2007, 2, 47.
13. Murugesan, S.; Park, T. J.; Yang, H.; Mousa, S.; Linhardt, R. J. Langmuir 2006, 22, 3461.
14. Lewis, A. L.; Tolhurst, L. A.; Stratford, P. W. Biomaterials 2003, 23, 1697.
15. Li, C. M.; Tang, Y. Q.; Armes, S. P.; Morris, C. J.; Rose, S. F.; Lloyd, A. W. Biomacromolecules 2005, 6, 994.
16. Xu, J. P.; Ji, J.; Chen, W. D.; Shen, J. C. J Control Release 2005, 107, 502.
17. Du, J. Z.; Tang, Y. Q.; Lewis, A. L.; Armes, S. P. J Am Chem Soc 2005, 127, 17982.
18. Chen, X. Y.; Armes, S. P. Adv Mater 2003, 15, 1558.
19. Narain, N.; Housni, A.; Lane, L. J Polym Sci Part A: Polym Chem 2006, 44, 6558.
20. Dalton, A. B.; Collins, S.; Razal, J.; Munoz, E.; Ebron, V. H.; Kim, B. G.; Coleman, J. N.; Ferraris, J. P.; Baughm, R. H. J Mater Chem 2004, 14, 1.
21. Xu, J. P.; Ji, J.; Chen, W. D.; Fan, D. Z.; Sun, Y. F.; Shen, J. C. Eur Polym Mater 2004, 40, 291.
22. Kong, H.; Gao, C.; Yan, D. Y. Macromolecules 2004, 37, 4022.
23. Tang, Q. G.; Ji, J.; Barbosa, M. A.; Fonseca, C.; Shen, J. C. Biomaterials 2003, 24, 4699.
24. Lobb, E. J.; Ma, I.; Billingham, N. C.; Armes, S. P. J Am Chem Soc 2001, 123, 7913.
25. Matyjaszewski, K.; Xia, J. H. Chem Rev 2001, 101, 2921.
26. Feng, W.; Brash, J.; Zhu, S. P. J Polym Sci Part A: Polym Chem 2004, 42, 2931.
27. Huang, W. J.; Taylor, S.; Fu, K. F.; Lin, Y.; Zhang, D. H.; Hanks, T. W. Nano Lett 2002, 2, 311.
28. Wuang, S. C.; Neoh, K. G.; Kang, E. T.; Pack, D. W.; Leckband, D. E. Adv Funct Mater 2006, 16, 1723.