http://dx.doi.org/10.5012/bkcs.2013.34.1.149

Synthesis, Characterization, and the Influence of Functionalized Multi-Walled Carbon Nanotubes with Creatinine and 2-Aminobenzophenone on the Gastric Cancer Cells

Hasan Tahermansouri,[*] Yaser Aryanfar, and Esmaeil Biazar[†]

Department of Chemistry, Ayatollah Amoli Branch, Islamic Azad University, P.O. Box 678 Amol, Iran
[*]E-mail: h.tahermansuri@iauamol.ac.ir
[†]Department of Biomaterials Engineering, Tonekabon branch, Islamic Azad University, Tonekabon, Iran
Received September 12, 2012, Accepted October 22, 2012

The chemical functionalization of carboxylated multi-walled carbon nanotubes (MWCNT-COOH) by creatinine (MWCNT-Amide) and latter modification with 2-aminobenzophenone for producing 1-methyl-9-phenyl-1*H*-imidazo[4,5-*b*]quinolin-2-amine (MWCNT-quino) have been investigated. All products were characterized by Fourier transform infrared spectroscopy, Raman spectroscopy, scanning electron microscope, elemental analysis, thermogravimetric analysis, derivative thermogravimetric and cellular investigations. The interesting point is that MWCNT-quino can be homogeneously dispersed in dimethylformamide and to some extent in ethyl alcohol without sonication. Also, MTT assay was used to examine the behavior of cell proliferation after 48 h of cell culture experiments. Cellular results showed high toxicity of MWCNT-quino on the cancer cells. These functionalizations have been chosen due to active sites of carbonyl and methylene groups in MWCNT-Amide and the creating quinoline derivative on the MWCNTs for future application.

Key Words : Carbon nanotube, Cancer cells, Functionalization, Toxicity

Introduction

The discovery of multi-walled carbon nanotubes (MWCNTs) in 1991[1] has generated extensive activity in most areas of science and engineering because of molecular electronics, sensoring, nerve cell stimuli, drug delivery, cancer therapy and chemical properties.[2-4] In particular, chemical functionalization of carbon nanotubes (CNTs) can modify their physical and chemical properties, leading to the improvement of their performance for specific applications. The chemical functionalization of CNTs which represent an emerging area in the research on nanotubes-based materials enables chemical moieties on their surface that can conjugate to anti cancer drugs by functional groups. The functionalized carbon nanotubes (f-CNTs) are believed to be very promising in the field of biological technologies. For example, Hu *et al.* have investigated the growth of neurons on functionalized multi walled CNTs.[3] Also, it have confirmed that gene expression through f-CNTs levels up to 10 times higher than those achieved with deoxyribonucleic acid (DNA) alone.[5] Thus, the extensive research has been focused on the functionalization of CNTs which can be cited fluorination,[6] carbene addition,[7] estrification[8] and amidation.[9-13] Also, several review articles of the functionalization of CNTs have been reported.[14,15]

In this current study, in addition developing the amidation of MWCNT with creatinine we have investigated the formation of 1-methyl-9-phenyl-1*H*-imidazo[4,5-*b*]quinolin-2-amine (quino) on the MWCNTs which were characterized by Fourier transform infrared spectroscopy (FT-IR), Raman, scanning electron microscope (SEM), thermo gravimetric analysis (TGA), derivative thermo gravimetric (DTG) and elemental analysis. Synthesis route of modified MWCNT-COOH was shown in Figure 1.

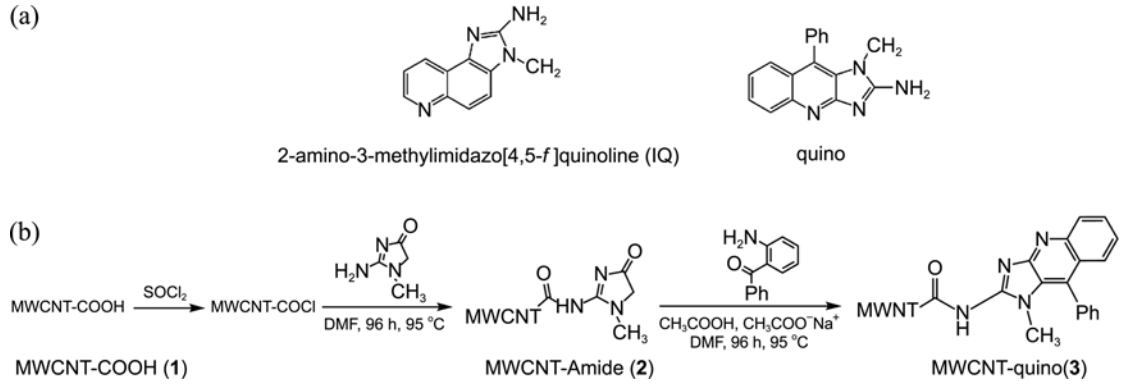


Figure 1. (a) 2-Amino-3-methylimidazo [4,5-*f*]quinoline (IQ) and quino structure (b) Synthesis route of modified MWCNT-COOH.

The structure of this compound, quino, is similar to 2-amino-3-methylimidazo [4,5-*f*]quinoline (IQ) which is the powerful animal carcinogen.[16] With this background, we have investigated the effect of modified MWCNTs on the cancer cells. Cellular results showed high toxicity of MWCNT-quino on the cancer cells.

Experimental

Materials and Characterizations. All reagents and solvents such as thionyl chloride ($SOCl_2$), creatinine, 2-aminobenzophenone, tetrahydrofuran (THF) and dimethylformamide (DMF) from Merck Chemical Inc. and MWCNT-COOH (%95 purity, 20-30 nm, Netvino Co., Ltd) were purchased and used as received. FT-IR spectrum was recorded using KBr tablets on a Thermo Nicolet Nexus 870 FTIR spectrometer. Raman spectra recorded on VARIAN-CARY 100 spectrometer. SEM was used to study the morphology of the MWCNTs. SEM measurement was carried out on the XL30 Philips Electron Microscope. Elemental analyses of C, H, N were performed with a SERIES (II) 2400 from Perkin Elmer Co. USA. The samples investigated by thermogravimetric analysis (NETZSCH TG 209 F1 Iris) in the N_2 (10 ^oC/min).

Preparation of MWCNT-Amide 2. 100 mg of the MWCNT-COOH were suspended in 15 mL $SOCl_2$ and 1 mL DMF. Then, the mixture was stirred at 70 ^oC for 48 h under reflux. Subsequently, the residual $SOCl_2$ was removed by reduced pressure distillation to yield the acylchloride-functionalized MWCNT (MWCNT-COCl). 80 mg of MWCNT-COCl were mixed with 150 mg of creatinine in 25 mL DMF and the reaction mixture was stirred at 90 ^oC for 96 h. Then, the mixture was cooled to room temperature, filtered and washed thoroughly with DMF, ethyl alcohol and THF.

Subsequently, the black solid was dried at room temperature for 8 h under vacuum condition.

Preparation of MWCNT-quino 3. 50 mg of the MWCNT-Amide was sonicated in 10 mL DMF for 15 minutes gave out a homogeneous suspension. Then, the mixture of 100 mg of 2-aminobenzophenone and 10 mL buffer (the mixture of 10 mL acetic acid and 0.2 gr sodium acetate) was add to the reaction mixture and it was stirred at 95 °C for 96 h. After cooling to room temperature, the reaction mixture was separated by centrifugation and washed thoroughly with DMF, ethyl alcohol and THF. Thus, the obtained solids were dried by vacuum for 6 h.

Cellular Study.

Materials: The following substances were obtained from the sources as indicated. Cell culture medium (RPMI1640), fetal calf serum, 0.25% trypsin with 1 mM ethylenediaminetetraacetate (EDTA), streptomycin sulfate, and penicillin G sodium, all from GIBCO (Grand Island, NY, USA) was obtained. Human gastric cancer cell line MKN45(NCBI No: C615) was provided by the Iranian pastor institute cell bank (Tehran ,Iran).

Cell Culture: MKN-45 was cultured in RPMI1640 supplemented with 10% fetal bovine serum and 1% L-glutamine, penicillin, and streptomycin. For these experiments, cells were seeded at a density of 1×10^5 cells/ml in 96-well plates. At least three time points are depicted for each assay.

Cell Proliferation: The MTT assay (Sigma) was used to evaluate the proliferative activity. Cells grown in 96-well plates were exposed to MWCNTs 1-3. Exposure to 50 μL RPMI1640 was used as a negative control and 50 μL dimethyl sulfoxide as a positive control. After 48 h, 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution at 0.5 mg/mL in Hank's balanced salt solution was added to each well and incubated for 4 h. Afterward, 150 μL of the MTT solution (10% Triton X-100 in 0.1 N HCl in anhydrous isopropanol) was added to each well. The resulting formazan crystals were solubilized in acidic isopropanol and quantified by measuring absorbance at 570 nm with micropleat reader (Rayto RT-6100). Data were calibrated to the appropriate calibration curve, as stated in Sigma protocols.

Statistics: All data were collected using EXCEL. Graphing and statistics were performed with SPSS11.5. Continuous variables were presented as mean ± S.D(x ± s). Data were analyzed using one-way anova to determine the difference among groups. Relative grown rate (RGR) of cells was calculated using the formula, RGR = OD of each group/OD of natural group × 100%. To determine the cytotoxic response of the MWCNTs to cells in culture, 6-graded toxicity and other analytic methods were used.

Results and Discussion

The Figure 2 presents the FT-IR spectrum of modified MWCNTs (a-c). In spectrum (a), the peak at 1530-1560 cm^{-1} is assigned to stretching mode of the C=C double bond that forms the framework of the carbon nanotube sidewall.[11] The appearance of the absorption peaks at 1721 and 1045 cm^{-1} in the IR spectra of MWCNT-COOH clearly indicates carboxylic groups on the MWCNTs.[9] The two bands at 2800-2950 cm^{-1} which are seen in all spectra are assigned to CH stretching of MWCNT-COOH defects. In the spectrum (b), the appearance of the two new peaks at 3100-3300 cm^{-1} (N-H and OH stretching modes) and 1662 cm^{-1} (C(=O)NH linkage) indicates that creatinine has been successfully

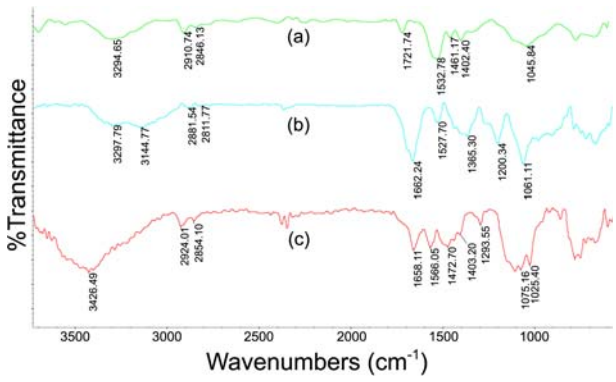


Figure 2. FT-IR spectra (after baseline correction) of MWCNT-COOH (a), MWCNT-Amide (b) and MWCNT-quino (c).

Table 1. Elemental analysis of modified MWCNTs **1-3**

MWCNTs	%C	%H	%N
1	94.28	0.42	0.00
2	89.44	1.18	3.79
3	90.60	1.51	4.21

attached onto the external surface of MWCNTs. From these results, it can be deduced that the formation of amide bonds can lead to the strong linkage of creatinine with carboxylic groups of MWCNT-COOH. Also, the peak at around 1690 cm^{-1} which is overlapped with C=O of nanotube can be related to C=O of creatinine. In the spectrum (c), the peak of C=O creatinine has been disappeared and the remarkable peak at around 1566 cm^{-1} are appeared which can be assigned to C=N bond. The peaks at around 1530-1580, 1400-1530, 1200-1380 and 1000-1100 cm^{-1} correspond to C=C stretching nanotube, aromatic ring modes, C-N and C-O stretching modes, respectively. Thus, FT-IR spectra confirm that MWCNT-COOH has been successfully modified by quinoline derivative.

Elemental analysis of modified MWCNTs **1-3** are shown in Table 1. Apart from the carbon values, the changes of atomic percentages H (1.18%) and N (3.79%) in **2** and H (1.51%) and N(4.21%) in **3** (as compared those in 1) indicated that **1** is functionalized. On the other hand, the increase of percentage of H and N for **3** in comparing to **1** and **2** confirms the formation of quinoline derivative. Based on these data coupled with the assumption that the atomic percentages of nitrogen and hydrogen were originated from the employed creatinine and 2-aminobenzophenone, we confirmed the functionalization of MWCNT-COOH.

The best evidence for the functionalization of MWCNTs is TGA results that provide quantitative information on the functionalization of MWCNTs. According to Figure 3(a), TGA graph of MWCNT-Amide display a gradual trend in decomposition from 240 to 360 °C with a weight loss of about 21.74% that can be assigned to the attached creatinine to CNT as comparing to thermogram itself. In TGA of creatinine, a weight loss between 200 and 270 °C is observable which probably can be assigned to decomposition of creatinine to creatine. Also, the TGA curve of MWCNT-quino presents one decomposition at around 470-590 °C with a weight loss of about 8.7%, probably coming from the

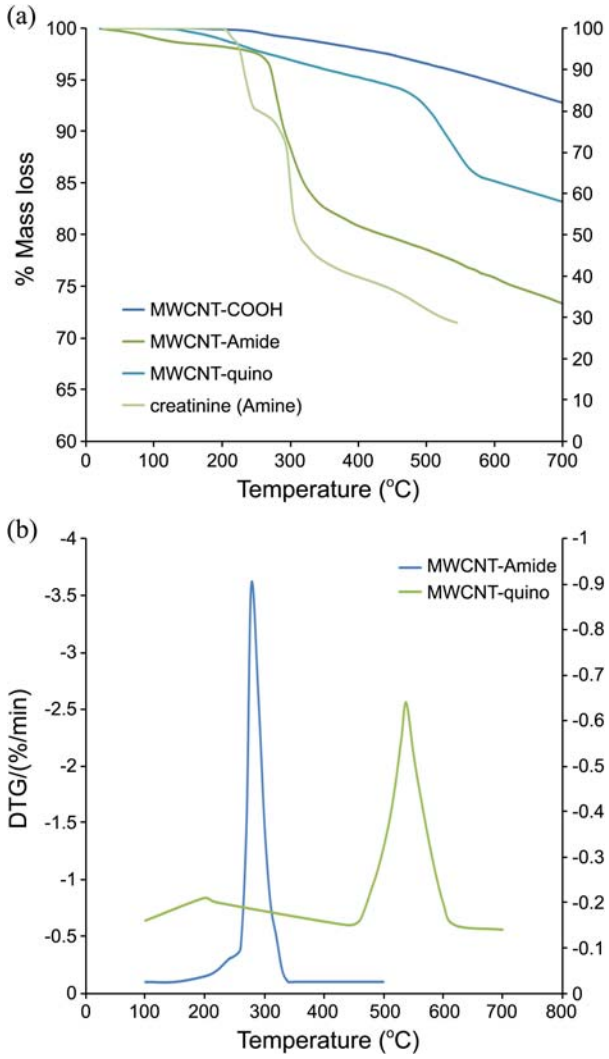


Figure 3. TGA (a) and DTG (b) curves of modified-MWCNTs in the N_2 (10 °C/min).

loss of the attached quinoline derivative groups to CNT (as compared with the TGA curve of MWCNT-Amide). These results indicate that there is one creatinine group for MWCNT-Amide per 31.3 and one quinoline derivative group for MWCNT-quino per 225 carbon atoms of MWCNT, respectively at 600 °C.

The DTG curve provides further evidence for covalent modification of MWCNTs (Figure 3(b)). The one major

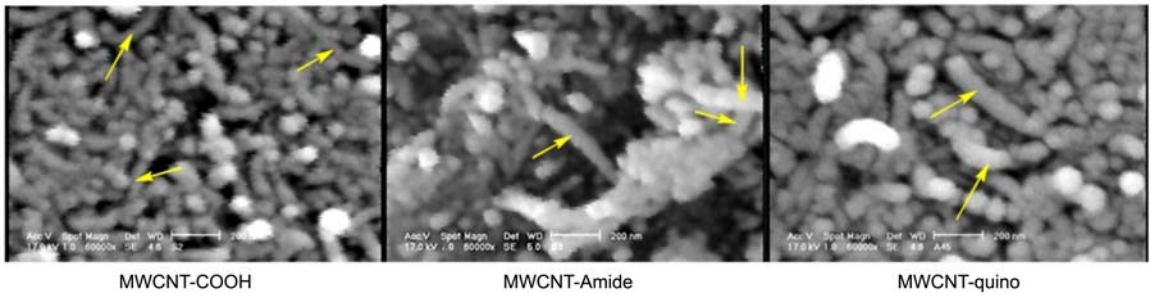


Figure 4. The SEM images of modified MWCNTs.

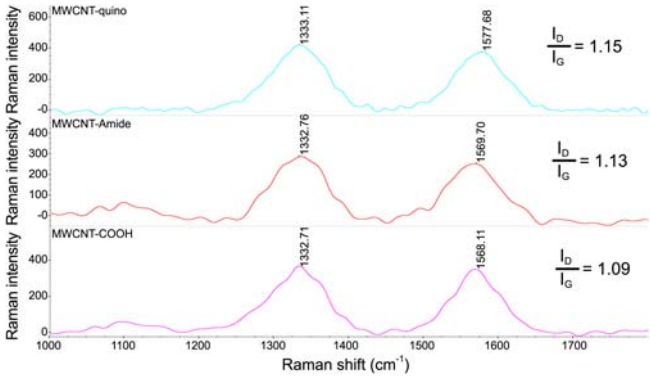


Figure 5. The raman spectra of modified MWCNTs. The baseline was corrected for the luminescence background.

peak at 278 ºC could be attributed to the loss of the creatinine groups bonded to MWCNT. On the other hand, the DTG curve of MWCNT-quino show one peak at 538 ºC which can be assigned to the loss quinoline derivative. Overall, these results successfully confirm the functionalization of MWCNT-COOH with these compounds.

In Figure 4, the SEM images present the surface morphology of the functionalized MWCNTs. These images show that the resulting samples have the morphology of nanotubes and these nanotubes are obviously different from MWCNT-COOH. As can be seen in the figure, the functionalized CNTs appear to be thicker compared to the MWCNT-COOH. On the other hand, in the MWCNT-Amide and MWCNT-quino images a tubular layer of uniform organic compounds (creatinine and quinoline derivative, respectively) is clearly present on the surface of the MWCNTs (the rough part) and the their diameters are slightly increased due to a covalently bonded creatinine and quinoline derivative on the surface of the MWCNT as compared to that of MWCNT-COOH.

The Raman spectrum which is a powerful tool for characterization of both MWCNT-COOH and modified MWCNTs are shown in Figure 5. Generally the G lines, which is tangential-mode peaks, are observed at 1560-1580 cm^{-1} and D lines, attributed to the defects and disorder-induced peaks, are observed at 1332 cm^{-1} [17,18] for MWCNT-COOH, MWCNT-Amide and MWCNT-quino. The disorder mode, attributed to sp^3 hybridized carbon atoms in the benzenoid framework of the carbon nanotube walls, is widely used as a measure of covalent sidewall functionalization.[19] It is obvious that (I_D/I_G) ratio increases from 1.09 in MWCNT-COOH to 1.16 in MWCNT-Amide and 1.18 in MWCNT-quino after functionalization. This increase in D band intensity is due to the covalent binding of the addends leading to many sp^3 defects in the sidewalls.[20] In other words, this indicates a partial destruction in the conjugation structure of the MWCNTs because of attaching creatinine and quinoline derivative.

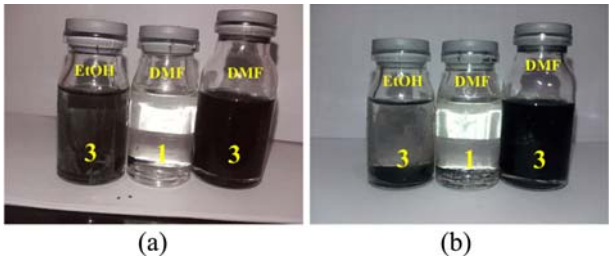


Figure 6. The dispersion images of MWCNT-COOH (1) and MWCNT-quino (3) in DMF and EtOH (1 mg/7 mL) after standing for (a) 2 h and (b) 3 months.

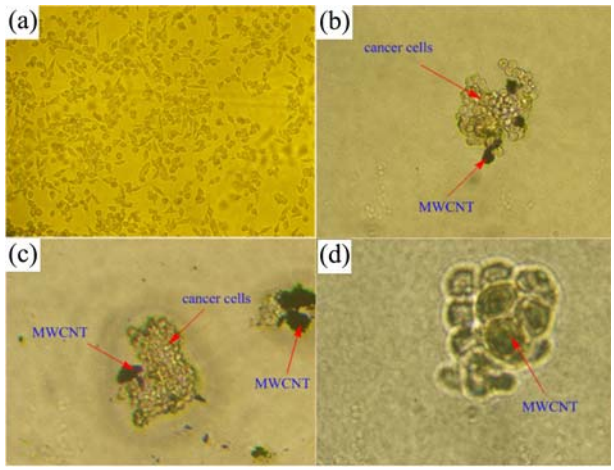


Figure 7. Cell growth on the samples. Control (a); MWCNT-COOH (b); MWCNT-Amide (c); MWCNT-quino (d).

A fair idea whether the modification on the carbon nanotubes has been achieved or not is dispersion test. Figure 6 presents a photograph of the dispersion of MWCNT-COOH and MWCNT-quino in DMF and EtOH. As it can be seen from Figure 6, MWCNT-COOH are insoluble in DMF while the MWCNT-quino can be directly dispersed in DMF (without sonication) homogeneously and no precipitation was found even after it was sealed for 3 months at room temperature. In EtOH, MWCNT-quino showed less dispersion after 2 h (Figure 6(a)) and it precipitated after 2 days (Figure 6(b)). These results indicate which MWCNTs was functionalized by quinoline derivative.

The effect of functionalized MWCNTs was investigated on toxicity of cancer cells. The used biological data in this study had anti-cancer activity against MKN-45. The structural features and biological activity of these compounds are listed in Figures 7 and 8. The Figures show targeting can-

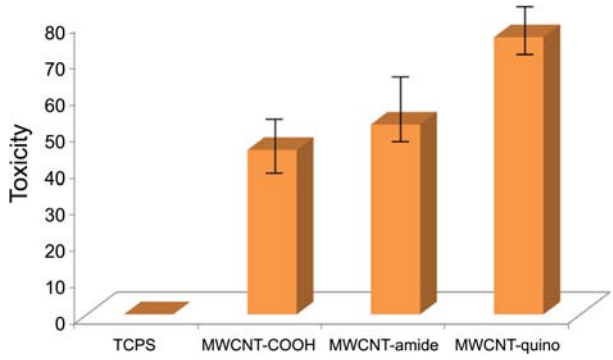


Figure 8. MTT assay for TCPS and modified MWCNTs in 48 h.

cerous cells by CNTs in particular MWCNT-quino. Figure 7(a) related to culture of cancerous cells on the TCPS (Tissue culture poly styrene) surface which show the cells of well adhesion and proliferation on the TCPS surface. Figure 7(b)-(d) related to effect of cancerous cells in the vicinity of modified MWCNTs as control sample. These show that the modified MWCNTs well diffuse into and on cancerous cells. Also, in Figure 7 a microscopy image was shown which diffusion of CNTs indicate into cancerous cells. For sample D , carbon nanotube completely diffused into the cells, but it are not occured for other samples and CNTs only agglomerate at around cells.

Figure 8 shows MTT assay for TCPS (control), MWCNT-COOH, MWCNT-Amide and MWCNT-quino. Almost, half of cells (44.18%) were killed by MWCNT-COOH. Also, the same result is obtained by the MWCNT-Amide (52.26%). However, the MWCNT-quino showed the high toxicity about 76% that can due to presence of heterocyclic and phenyl groups. We dictate which concentration of samples were considered similar to each other (100 μg/mL). These results was demonstrated that MWCNT-quino (D) is a powerful agent for cancer cells which it lead to kill cancer cells about 76%.

Conclusion

We have firstly demonstrated the anti-cancer agents by functionalization of MWCNTs. We have designed and synthesized novel derivatives of quinoline that have potential use for cellular toxicity especially cancerous cells. The reported results demonstrate the functionalization of MWCNT by a quinoline derivative. Functional groups attached to the surface of CNTs are able to modify the stacking and solvation properties of carbon nanotubes. Cellular results demonstrated that the MWCNT-quino is a more toxic agent compared to other samples for cancer cells and can be used as a toxic material for chemotherapy.

Acknowledgments. The financial and encouragement support provided by Research vice Presidency of Ayatollah Amoli branch, Islamic Azad University and Iranian Nanotechnology Initiative (Govt. of Iran).

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