The enhancement of neural growth by amino-functionalization on carbon nanotubes as a neural electrode

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1. Introduction

Carbon nanotubes (CNTs) exhibit many excellent physical and chemical properties, including high conductivity, high mechanical strength, large surface areas, flexibility, biocompatibility and biological inertness (Bardi et al., 2009; Krishnan et al., 1998; Lobo et al., 2008a,b; Shoval et al., 2009; Sorkin et al., 2009). High conductive and mechanical CNTs can activate resident immune cells in neural tissues (Kotov et al., 2009). The high surface area of CNTs can increase charge injection capacity and decrease the interfacial impedance with neuronal cells. CNTs also can provide excellent properties for interfacing with neural systems in the applications of durable, biocompatible, and powerful neuroprosthetic devices (Kotov et al., 2009). Besides, CNTs can promote cell attachment, growth, and long-term survival of neurons (Cellot et al., 2009) and CNT substrates can even boost neural electrical signals (Lovat et al., 2005). Some researches indicated that CNT electrode can potentially be used for retinal implants, network repair, and neuro-welding applications (Ben-Jaco and Hanein, 2008; Sorkin et al., 2009). In addition, CNTs are not biodegradable, and they could be used as implants for long-term extracellular recording (Hu et al., 2004). The typical neural electrodes have been fabricated from silicon (Ben-Jaco and Hanein, 2008; Lobo et al., 2008a,b; Zhang et al., 2005), tungsten, stainless, gold (Gabay et al., 2007; Keefer et al., 2008) and flexible substrates (Hsu et al., 2010; Lin and Lee, 2009), nevertheless, they often show poor contact which result in more noise and fluctuation in recordings (Keefer et al., 2008). Therefore, CNTs can be a substrate for better electro-chemical interface and be implanted in the human brain for extracellular recording (Biran et al., 2005; Minnikanti et al., 2009). However, the hydrophobic surface of CNTs is currently a barrier for biological applications, and it requires specific functional groups to make CNT surface more hydrophilic (Xu et al., 2008; Zhang et al., 2008). The properties of CNTs can be further enhanced through surface modification. Special treatments can be applied to facilitate novel interfacing. CNTs may be instrumental in establishing the coupling mechanism between cells and surface interactions (Kotov et al., 2009). Some researches suggested that the functionalization of MWCNTs is necessary to increase cell growth and viability (Correa-Duarte et al., 2004; Lobo et al., 2008a,b).
Fig. 1. Process flow of MWCNT formation and MWCNT amino-functionalization. (a) MWCNTs grew on the substrate. (b) Carboxylic groups formed on the MWCNT surface. (c) Acyl-chloride groups formed on the MWCNT surface. (d) Amino groups formed on the MWCNT surface.

Many methods for modification of CNT surface have been studied. Jan et al. (2009) and Keefer et al. (2008) reported the coating of polypyrrole (PPy), polyelectrolyte (PE) and poly(3,4-ethylenedioxythiophene) (PEDOT) to improve electrical properties. These were attributed to that a microelectrode coated with CNTs might exhibit low impedance, high charge transfer, and biocompatibility. Gheith et al. (2006) reported that CNTs could be incorporated into the multilayers formed by poly(N-cetyl-4-vinylpyridiniumbromide -co-N-ethyl-4-vinylpyridiniumbromide-co-4-vinylpyridine) with high conductivity to electrically stimulate excitable neuronal cells. Sorkin et al. (2009) and Ben-Jaco and Hanein (2008) also reported CNTs treated by O2 plasma could exhibit better hydrophilic surface for neuronal growth. It is suitable for interfacing with a single cell at very high level of fidelity and also able to pattern neuronal circuits. Therefore, understanding the reaction of the interface between neurons and CNTs could be achieved. CNTs were also treated by UV-ozone to improve hydrophilicity, biocompatibility, interfacial impedance and adhesion, as reported by Hsu et al. (2010) and Sham and Kim (2006). Their results indicated good biocompatibility of UV-ozone modified CNT substrates for neuronal growth. Gailllard et al. (2009) and Mazzatenta et al. (2007) reported that the treatment for CNTs by 1,3-dipolar cycloaddition reaction in dimethylformamide (DMF) and peptides improves cell-adhesion. This study provided comprehensive evidence on the biocompatibility of functionalized MWCNTs with different cell types (splenocytes and neurons). Malarkey et al. (2009) and Zhao et al. (2005) reported that they could improve water-soluble property and biocompatibility by covalently attaching poly(aminobenzene sulfonic acid) (PABS) and polyethylene glycol (PEG) onto CNTs. They also demonstrated that CNT substrates have advantages for neuronal growth in vivo, preparation of composite materials, and biological research. Besides, Hu et al. (2004) modified the surface of CNT on glass substrate by poly-m-aminobenzene acid (PABS) for brain recordings. The results indicated that chemically modified CNTs which control the neurite outgrowth could be implemented clinically. Furthermore, neuronal growth was controlled by functionalizing MWCNTs with different functional groups, in which it was found that neurons grown on positively-charged MWCNTs exhibited more neurite growth and branching (Kotov et al., 2009). Positively charged surfaces as a polar surfaces reveal more neurite branching than that of non-polar surfaces.

Instead of growing CNTs at high temperatures (Gabay et al., 2007; Lobo et al., 2008a,b; Sorkin et al., 2009), dispersing or coating CNTs onto substrates (Hu et al., 2004; Keefer et al., 2008), in this work we synthesized self-aligned multi-walled carbon nanotubes (MWCNTs) on Si-based substrates at low temperature (400 °C) developed in our groups (Hsu et al., 2009; Su et al., 2010a). Furthermore, amino-functionalization was modified directly onto CNTs by 1,4-diaminobutane after CNTs growth. Finally, detailed characterization analyses were carried out in this work, including electrochemistry, biocompatibility and neuron signal measurement. It is the first time to report the direct growth at low temperature (400 °C) and direct modification of CNTs grown on Si-based substrates by 1,4-diaminobutane, which exhibit the advantages of improving neuron attachment for neural signal detection and being able to be friendly implemented in IC fabrications.

2. Materials and methods

2.1. MWCNT electrode fabrication and modification

Fig. 1 shows the process flow of MWCNT formation and MWCNT amino-functionalization. After series of process optimization, the MWCNTs were synthesized on Ni (5 nm)/Ti (20 nm)/Au (200 nm)/Cr (20 nm)/SiO2 (200 nm)/Si substrates using 5 nm-thick Ni catalyst and C2H2 (60 sccm)/H2 (10 sccm) process gases at 10 Torr by chemical vapor deposition (CVD) at a low temperature (400 °C) (Fig. 1a). Microwave treatments at 1100 W for 3 min were used to enhance the adhesion between MWCNTs and substrates by forming nickel carbides (Su et al., 2010a). Fig. 1b shows that as-grown MWCNTs were treated with H2O plasma to form the carboxylic groups on MWCNT surface (MWCNT–COOH), which will be published.
Fig. 2. XPS analyses show (a) C 1s spectrum of as-grown MWCNTs, (b) C 1s spectrum of MWCNT–COOH, and N 1s spectra of MWCNTs modified by (c) 0.2 wt%, (d) 1 wt%, (e) 2 wt%, (f) 3 wt% 1,4-diaminobutane, respectively. (g) Quantities of amine (NH₂) groups modified on the MWCNT surface at various concentrations of 1,4-diaminobutane.

by our co-authors (Chen et al., 2010). Carboxylic MWCNTs were then placed on the specimen kit containing chemical solutions. The carboxylic MWCNTs were refluxed in thionyl chloride (SOCl₂, 98%) under Ar ambient for 18 h at room temperature (RT) to generate an acyl-chloride derivative (MWCNT–COCl), as shown in Fig. 1c. The resulting MWCNT–COCl were added to a mixture of 1,4-diaminobutane (2 wt%), dried toluene, and small amount of triethylamine. Then the mixture with MWCNT–COCl was refluxed for 9 h at room temperature under Ar ambient. Finally, amino groups were developed on the MWCNT surface (Fig. 1d).
Fig. 3. SEM images of (a) as-grown MWCNTs, and MWCNTs modified by (b) 0.2 wt%, (c) 1 wt%, and (d) 2 wt% 1,4-diaminobutane. The insets were their contact angle analysis, respectively.

2.2. Neural signal measurement

The crayfish was anesthetized in 4°C water bath, and the nerve cord was dissected and pinned dorsal side up on Sylgard 184 (Dow Corning, Midland, Michigan, USA) in a Petri dish. The preparation was kept in crayfish saline (Van Harreveld, 1936) containing 210 mM NaCl, 15 mM CaCl₂, 5.4 mM KCl, 2.6 mM MgCl₂, and 5 mM HEPES (all purchased from Sigma–Aldrich, St. Louis, Missouri, USA) at pH = 7.4.

Electrophysiological recording was performed according to procedures described previously (Bahar, 2003; Rodriguez-sosa et al., 2007). A suction glass pipette filled with crayfish saline, an Au electrode and a MWCNT electrode were all placed on the ventral surface of the 2nd segment of abdominal nerve cord of crayfish to record caudal photoreceptor (CPR) spikes. Higher frequency of CPR spikes appeared after the 4th second; these spikes were induced by the light illuminating (using light emitting diode, white light, 5 V, 30 mA, 1 s) on the dendritic tree of CPR cell in the 6th ganglion.

2.3. Cell culture

Primary hippocampal neuronal cells were harvested from rat fetuses for sixteen days according to a previously reported procedure (Cheng et al., 2009). Before culturing neuron cells, all materials were subjected to a sterilization process, with the substrates immersed in alcohol for 30 min and then rinsed in DI water 3 times. After culturing for 3 days, cells underwent treatments with (+) and without (−) 5 μM cytosine-β-d-arabinofuranoside (Arc). Morphologies of neurons and neurite outgrowth branches were analyzed after cell culturing for 16 days. All cells were observed under confocal laser scanning microscopy (CLSM) with a 488 nm excitation wavelength, and cell marking were carried out by using βIII tubulin as a neuronal cell marker, glial fibrillary acidic protein (GFAP) as a glial cell marker, and 4,6-diamidino-2-phenylindole (DAPI) as a nuclear marker. For each fluorescent image in the biocompatibility tests, at least 3 randomly selected fields of view (each with an area of 0.7 mm²) per sample (area = 1 cm²) were analyzed, and 3 samples were prepared in different experiments for each cell culturing.

2.4. Characterization

X-ray photoelectron spectroscopy (XPS) was used to characterize the chemical functionalization of CNT surface. Scanning electron microscopy (SEM, JEOL 6500), high-resolution transmission electron microscopy (HRTEM, JEOL JEM-2010) and micro-Raman spectra (HORIBA HR800) were utilized to analyze the degree of graphitization of CNTs and identify the MWCNT structures. Frequency-dependent changes between the impedance of as-grown and amino-functionalized MWCNT electrodes were characterized by an Aligent 4284A system. The 10 mV sinusoidal signals with various frequencies (20–100 kHz) were applied to MWCNT electrodes in a phosphate buffered saline (PBS) solution using Ag/AgCl coil as a reference electrode. Cyclic voltammetry (CV) measurements were also conducted to determine the electrochemical properties of as-grown and amino-functionalized MWCNTs (AF-MWCNTs) electrodes. The surface wettability of MWCNTs was measured by a contact angle system. Each data point in this study was obtained from at least 3 samples (n = 3) in different experiments.

3. Results

3.1. Physical characterization of neural electrode

The XPS spectra in Fig. 2a and b provides evidence of the carboxylic group bonding on the surfaces of as-grown and H₂O plasma-treated MWCNTs, respectively. The C 1s XPS spectrum can be fitted to five Gaussian–Lorentzian peaks, which are attributed to sp² hybridized carbons sp²-C (~284.5 eV), sp³-C (~285.4 eV), C–O (~286.2 eV), hydroxyls C–OH (~287.6 eV), and carboxyls HO–C=O (~288.9 eV) (Datsyuk et al., 2008; Lee et al., 2001, 2008). These fig-
Fig. 4. Raman spectrum analyses of (a) the trend chart of the D-band to G-band intensity ratio (I_D/I_G). (b) Raman spectra of as-grown MWCNTs, MWCNTs modified by SOCl₂, and MWCNTs modified by 0.2 wt%, 1 wt%, and 2 wt% 1,4-diaminobutane. The high-resolution TEM images of (c) as-grown MWCNTs, and (d) 2 wt%-AF-MWCNTs. 

ures show that the total percentages of C–O, C–OH, and HO–C = O bonds in MWCNTs after H₂O plasma-treated is higher than that of as-grown MWCNTs, similar to the results reported earlier (Su et al., 2010b). It is possible that the formation of hydrophilic chemical bonds such as C–OH, C=O and OH–C=O on the outermost surface of the MWCNTs (Li et al., 2007) has been enhanced by H₂O plasma treatment.

On the other hand, Fig. 2c–f shows the N 1s XPS spectra of AF-MWCNTs treated with concentrations of 1,4-diaminobutane 0.2 wt%, 1 wt%, 2 wt%, and 3 wt%, respectively. The binding energies at 399.9 eV and 400.8 eV on the N 1s XPS spectrum correspond to the nitrogen in amide (–CONH–) and amine (–CNH₂–) groups, respectively (Ramanathan et al., 2005; Shen et al., 2007). Furthermore, three identical samples collected from each 1,4-diaminobutane concentration were prepared for the quantitative analysis of amine groups at different concentrations of 1,4-diaminobutane (Fig. 2g), showing that the highest quantities of amine groups occur to the 2 wt% 1,4-diaminobutane treated MWCNTs (2 wt%-AF-MWCNTs). However, the quantity of amine groups on MWCNTs decreases as 1,4-diaminobutane concentration increases to 3 wt%, which was mostly due to the cross-linking between 1,4-diaminobutane chemical compounds that degrades the amino group formation. Table S1 in Supplementary data summarizes the quantities of amide (–CONH–) and amine (–CNH₂–) groups on MWCNT surface modified by various concentrations of 1,4-diaminobutane.

Fig. 3a shows the 45°-tilted top-view SEM of as-grown MWCNTs with the side-view SEM shown in the inset. It can be observed that dense MWCNTs (density: 4 × 10¹⁰ cm⁻²) with a length of 1 μm are vertically aligned on the substrate. Fig. 3b–d shows SEM images of AF-MWCNTs treated by 1,4-diaminobutane with concentrations of 0.2 wt%, 1 wt%, and 2 wt%, respectively. These SEM images show that some AF-MWCNTs have been bundled together. Results of contact angle tests (the insets of Fig. 3a–d) indicate that the AF-MWCNTs are hydrophilic. Therefore, AF-MWCNTs tend to collapse into microbundles when the solvent evaporates, similar to the result reported by Correa-Duarte et al. (2004), in which they also mentioned that hydrophilic CNT surface was able to increase surface area for cell attachment.

Fig. 4a shows the Raman spectra of as-grown, acyl-chloride and amino-functionalized MWCNTs. All spectra exhibit two major peaks at 1330 cm⁻¹ (D-band) and 1594 cm⁻¹ (G-band), indicating amorphous and graphitized carbon, respectively. It can be observed that the functional modification of MWCNTs slightly influenced the shifts of the D-band and G-band. Fig. 4b shows the dependence of D-band to G-band intensity ratio (I_D/I_G) on the intensity of amino groups in MWCNTs treated with different concentrations of 1,4-diaminobutane. There is less than 10% reduction in I_D/I_G for the 1,4-diaminobutane treated MWCNTs compared to as-grown MWCNTs which exhibit the lowest intensity. The slight decrease of I_D/I_G ratio indicates a minor structural change of MWCNTs after the treatment using different concentrations of 1,4-diaminobutane (Li et al., 2007). According to the results of XPS analysis and Raman spectroscopy, we can confirm that amino groups have been successfully grafted on the MWCNTs surface. High-resolution transmission electron microscopy (HRTEM) was also used to analyze the as-grown and 2 wt%-AF-MWCNT structures. Fig. 4c confirms that the as-grown CNT is a multi-walled carbon nanotube. Compared with as-grown MWCNTs, the AF-MWCNTs treated with a concentration of 2 wt% 1,4-diaminobutane show negligible change on the MWCNT surface (Fig. 4d).

3.2. Electrochemical characterization and neural signal recording of neural electrode

A good recording electrode for nerve tissue must provide good electrical conduction. This requires low impedance at around 1 kHz, which is a typical frequency for neural activity. Fig. 5a shows the
Fig. 5. The (a) interfacial impedance per unit area measured by EIS. (b) CV data of as-grown MWCNTs and AF-MWCNTs and (c) their capacitance per unit area. (d) The impedance per unit area of 2 wt% 1,4-diaminobutane modified CNT electrodes versus shelf-life time (stored in air environment). The extracellular recording of the caudal photoreceptor (CPR) on the activated axon of a crayfish using electrodes made of (e) 2 wt%-AF-MWCNTs, (f) suction glass pipette, and (g) Au.

electrochemical impedance spectroscopy (EIS) of as-grown and amino-functionalized MWCNTs in PBS solution. The impedances of as-grown MWCNTs, 0.2 wt%, 1 wt%, and 2 wt% 1,4-diaminobutane-modified MWCNTs were about 0.37, 0.32, 0.27, and 0.19 kΩ mm$^{-2}$, respectively. This result indicates that impedance of AF-MWCNTs was lower than that of as-grown MWCNTs. Besides, the MWCNTs modified with higher concentration of 1,4-diaminobutane (2 wt%) show the lowest impedance compared to those modified with 0.2 wt% and 1 wt% 1,4-diaminobutane. This can be attributed to that the surface of AF-MWCNTs was more hydrophilic than that of as-grown MWCNTs, according to the results of contact angle between the MWCNT surface and PBS solution (see insets of Fig. 4a).

Fig. 5b shows the CV measurements in PBS solution of as-grown and AF-MWCNTs treated with different concentrations of 1,4-diaminobutane at a scan rate (v) of 100 mV s$^{-1}$. The formula used to derive the interfacial capacitance ($C_i$) related to the surface area of the electrode was $C_i = (Δi/2v)$, in which $Δi$ is the current deviation between positive and negative voltage. Based on the calculations above, the interfacial capacitance for as-grown MWCNTs is about 2.1 F mm$^{-2}$ (Fig. 5c). This value is more than ten times higher than that of conventional noble-metal electrodes ($\sim$0.2 F mm$^{-2}$) (Nguyen-Vu et al., 2006). Fig. 5c also shows the $Δi$ of MWCNTs increases by about 6.7, 7, and 10 times, corresponding to the interfacial capacitance of 14.1, 15.5 and 22 F mm$^{-2}$, after the modification with 0.2 wt%, 1 wt% and 2 wt% 1,4-diaminobutane, respectively. Above results indicate that 2 wt%-AF-MWCNT can provide better sensitivity and facilitate their application for neural recording.
The durability of AF-MWCNTs was also tested. Fig. 5d shows the impedances monitoring of AF-MWCNTs treated by 2 wt% 1,4-diaminobutane and stored in air ambient for up to six months. From EIS measurement, the impedance of 2 wt%-AF-MWCNTs electrodes measured in PBS solution only showed slight change over six months of tracking. These results revealed that the amine groups were grafted on MWCNT surface strongly and reduced the impedance fluctuation of MWCNT electrode. It indicates that 2 wt%-AF-MWCNTs can be useful for long-term neural recording application.

The capability of the MWCNT electrode was also demonstrated by its employment on recording the neural signals of caudal photoreceptor (CPR). Fig. 5e and f shows the simultaneous extracellular recording on the axon of the CPR interneuron of crayfish using a 2 wt%-AF-MWCNT electrode and a suction glass pipette. The action potentials measured by a 2 wt%-AF-MWCNT electrode and suction pipette exhibit low noise and fluctuation, compared to that of an Au electrode (Fig. 5g). The signal-to-noise ratio of 2 wt%-AF-MWCNT electrode, suction glass pipette and Au is 126, 23 and 18, respectively.

3.3. Cell culture for biocompatibility tests of neural electrode

For the biocompatibility tests, the samples with cells cultured on glass, as-grown MWCNTs, and 2 wt%-AF-MWCNTs were observed by confocal laser scanning microscopy (CLSM). Results show that

![Fig. 6. Fluorescent images of hippocampus neuronal and glial cells cultured on the surfaces of glass, as-grown MWCNTs and 2 wt%-AF-MWCNTs with (+) and without (−) a PLL coating and also treated with (+) and without (−) Arc. These images depict the cell growth with (a) +PLL–Arc, (b) −PLL–Arc, (c) +PLL+Arc, and (d) −PLL+Arc on glass; (e) +PLL–Arc, (f) −PLL–Arc, (g) +PLL+Arc, and (h) −PLL+Arc on as-grown MWCNTs; (i) +PLL–Arc, (j) −PLL–Arc, (k) +PLL+Arc, and (l) −PLL+Arc on 2 wt%-AF-MWCNTs. (m) Ratio for the occupied area of neurons to the total occupied area of neuronal and glial cells. (n) Average quantities of neuron attached on the surface of glass, as-grown MWCNTs and 2 wt%-AF-MWCNTs. Neuron cells were stained with βIII-tubulin (Green), glial cells were stained with Dil (red), and nuclei were stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
co-cultured neuronal (cells in green color)/glial (cells in red color) cells (−Arc) could grow on the surface of glass no matter with (+) and without (−) poly-L-lysine (PLL) coating (Fig. 6a and b). Fig. 6c shows that cultured neuronal cells (+Arc, which curtails the growth of glial cells) could grow well on the glass with PLL coating, while barely grew on that without PLL coating (Fig. 6d). Fig. 6e and f shows that co-cultured neuronal and glial cells (−Arc) could grow on surface of as-grown MWCNTs no matter with and without PLL coating. However, cultured neuronal cells (+Arc) barely grew on the surface of as-grown MWCNTs no matter with or without PLL coating (Fig. 6g and h). On the other hand, co-cultured neuronal/glial cells (−Arc) could grow well on the 2 wt%–AF–MWCNT surface no matter with and without PLL coating, as shown in Fig. 6i and j. Especially, cultured neuronal cells could grow on the 2 wt%–AF–MWCNT surface no matter with or without PLL coating (Fig. 6k and l).

A software of MATLAB was used to calculate the area occupied by neuronal cells through quantifying the ratio of neuronal-cell number to that of total cells (both neuronal and glial cells) for all samples of co-cultured neuronal/glial cells (Fig. 6m). Results show that 2 wt%–AF–MWCNTs without PLL coating exhibit the highest percentage of neuronal cells, though the difference on the percentage of neuronal cell in Fig. 6m is not significant. The quantities of cultured neuronal cells grown on various surfaces are compared in Fig. 6n, showing that the highest quantities of neuronal cells were grown on 2 wt%-AF–MWCNTs with PLL coating. According to above results, the neuronal cells growth on the 2 wt%-AF–MWCNT electrode, compared to that on the as-grown MWCNT electrode, was improved even without PLL coating.

4. Discussion

Raman spectra show that the D-band and G-band of AF–MWCNTs were slightly influenced shifted. This is because chlorine atoms act as electron retracting groups with respect to NTs, causing a strong negative inducting effect (Shen et al., 2007). Therefore, the amino functional groups on AF–MWCNTs that replace chlorine atoms can conduct the opposite charge transfer from the lone pair of nitrogen to the NTs to cause G-band D-band shifts (Shen et al., 2007). Compared with as-grown MWCNTs, the slight decrease of \( I_D/I_G \) ratio indicates a minor structural change of MWCNTs after 2 wt% 1,4-diaminobutane modification accordance to XPS data. The results show that AF–MWCNT electrodes exhibit great potential for extracellular neural recording without the need of PLL coating.

The N 1s XPS spectra show that MWCNT surfaces can be successfully modified with amino groups by 1,4-diaminobutane. The hydrophilic surface of MWCNTs can decrease impedance from 0.37 to 0.19 k\( \Omega \) mm\(^{-2} \), which is attributed to the increase of interfacial capacitance from 2.1 to 22 F mm\(^{-2} \). Durability tests show that 2 wt%–AF–MWCNTs can last for six months in air ambient with only slightly change (<20%) in impedance. The neural recording of crayfish shows that 2 wt%–AF–MWCNTs can provide better capability on detecting action potentials of CP interneuron compared to sputtering pipette from the evidence of a higher \( S/N \) ratio (126 versus 23). Biocompatibility tests show that neuronal cells adhere well on the surface of 2 wt%-AF–MWCNTs without the need of PLL coating, which is mostly attributed to the positive charge of formed of \( \text{NH}_2 \) that promotes the adhesion and growth of neuronal cells.

Acknowledgments

This work was supported by National Science Council under project number NSC96–2627–E–007–002. The authors would like to thank cMEa team at NTHU for providing suggestions and help, as well as my group members for helpful discussion at NTHU. ESS at National Tsing Hua University for E-gun system supports and NFC at National Chiao Tung University for TEM supports.

Appendix A. Supplementary data


References