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ARTICLE TYPE

Enhanced Oxidized Regenerated Cellulose with Functionalized Multiwalled Carbon Nanotubes for Hemostasis Applications

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Oxidized Regenerated Cellulose (ORC) has been modified by incorporating aminated MWCNTs (MWCNT-NH_2)s. The pristine MWCNTs (pMWCNTs) were aminated which introduced the aromatic amine groups on the side wall of MWCNTs. For modification of neat ORC, the MWCNT-NH_2s was reacted with neat ORC. To explore the origin of this behavior, amination of MWCNTs, dispersion of MWCNT-NH_2s in the ORC matrix and their interfacial interactions were investigated by SEM, FT-IR and XPS. The analytical results show that during functionalization of MWCNTs, the amine groups grafted on the surface of MWCNTs. In addition, the FT-IR and XPS results revealed a relatively strong interaction existed between aminated MWCNTs and the ORC macromolecules. The hydrophilicity test results revealed significant increment in water uptake of MWCNT-NH_2s/ORC composites with increasing the concentration of MWCNT-NH_2s in composites. The haemostatic evaluation of MWCNT-NH_2s/ORC composites on rabbits shows that the aminated MWCNTs increases the rate of blood stopping and hence decreases the blood loosing from injured sites.

Keywords: Oxidized Regenerated Cellulose; Functionalized Carbon Nanotubes; Cross linking; Hemostasis,

1. Introduction

The multiwalled carbon nanotubes (MWCNTs) have attracted a great deal of interest in both science and engineering fields since they were discovered[1]. Their small diameter and large aspect ratio offers additional advantages for composite applications[2]. In the past few years, MWCNTs have been incorporated into a wide range of polymer matrices for various functional applications[3]. Among the Nano-medicine technology carbon nanotubes (CNTs) have recently emerged as a new option which analyzes the potential through possible toxicological implications in the field of medicine and nanopharmaceutics[4]. CNTs have recently gained popularity as potential drug carriers, therapeutic agents and for applications in diagnosis. Therefore, in a very short time, CNTs have become the focus of attention by scientist in a wide variety of disciplines. Application of CNTs in diagnosis and therapy of dreadful diseases is a field of current interest[5].

Due to the van der Waals attraction between the CNTs and their large surface area, the pristine MWCNTs (pMWCNTs) tend to form agglomerates during the preparation of composites with the polymers. Therefore, the MWCNTs dispersion in the polymer matrix is of great concern. Furthermore, in the case of the preparation of MWCNTs/polymer composites by solution-mixing, homogenous CNTs dispersion or MWCNT solubilization in solvents is still a big challenge as the MWCNTs are amphiphobic, that is, they repel common polar and nonpolar solvents. During the past several years, the surface modification of MWCNTs by either noncovalent or covalent functionalization methods has been used to improve the solubility or dispersion of MWCNTs in solvents or polymers[6]. The MWCNTs, which have been used as reinforcing fillers in polymeric biomaterials, will dramatically improve the materials' mechanical strength and simultaneously endow them with electric conductivity that may provide electrical stimulation for tissue engineering constructs[7]. The use of MWCNTs in vivo requires appropriate functionalization to reduce toxicity and non-specific binding.

As the most abundant renewable resource, cellulose can be converted into derivatives and regenerated fibers and films, as well as various functional materials[8]. The hemostatic products that made from different materials such as cellulose ether often come in thin slices, gelatin and collagen are often cavernous. Commercial Surgicel absorbable hemostatic agent has been widely applied in various surgeries and played an important role on stopping the bleeding[9]. Although this hemostatic material is broadly applied due to its excellent properties, the commercial Oxidized Regenerated Cellulose (ORC) has also shown several inherent disadvantages. For example, the hemostatic property of this material is relatively poor and has a low biodegradability. The ORC materials are made into gauzes or multi-layered filaments since it has great toughness and isn't readily dissolved in water or common organic solvents (Bagheri et al. 2008[10]; Quan et al. 2010[11]; Richard et al. 2002[12]; Ruan et al. 2004[13]). Surgicel® is currently one of the most widespread applied hemostatic materials in the world which its major component is ORC

(Breech et al. 2000[14]; Ryšavá et al. 2003[15]; Pedro et al. 2010[16]).

In this work, the prepared ORC films and fibers were reinforced with CNTs. The Multiwalled carbon nanotubes (MWCNTs) were used simply because of their cost advantage compared to singlewalled carbon nanotubes (SWCNTs)[17]. For this purpse, the ORC fibers and films were prepared with nitrogen dioxide(NO_2)/carbon tetrachloride(CCl_4) oxidation system. The MWCNTs, at first aminated and then introduced in ORC samples. To improve the covalen bond between aminated MWCNTs and ORC the 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC), Nhydroxyl-succinimide (NHS) and glutamic acid as cross linking bridges was carried out to fabricate a novel aminated MWCNTs/ORC nanocomposite. The EDC is a zero-length crosslinking agent used to conjugate carboxyl to amino groups. The NHS can improve the efficiency of EDC coupling reactions between carboxyl and amine groups. To investigate the effect of functionalization of MWCNTs, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) has been carried out. The functional groups on the sidewall of MWCNTs were monitored by Fourier transform infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy (XPS). The water uptake of prepared samples were determined by immersing in deionized water at room temperature.

2. Experimental

2.1 Materials

Pristine MWCNTs (pMWCNTs) were purchased from the Boyu Gaoke Co, Beijing, China. The diameter of MWCNTs is 10-20 nm, length is 10-30 μm and special surface area is higher than 200 $m^2 g^{-1}$. The p-amino benzoic acid, poly phosphoric acid and phosphorus penta oxide (P_2O_5) were purchased from Kermel of China as analytic reagent.

Regenerated cellulose filaments, used as the starting material, were obtained from Xinxiang City, Henan Province, China. Nitrogen dioxide (AR, 99.99%, w/w) was purchased from Summit Specialty Gases Co., Ltd., Tianjin City, China. Carbon tetrachloride (AR, 99.5%) and sodium hydroxide (AR, 96%, w/w) were supplied by Shuang Shuang Chemical Co., Ltd., Yantai, China. Ethanol (AR, 99.7%, w/w) was purchased from Fu Yu Chemical Co., Ltd., Tianjin, China. 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC) and Nhydroxyl-succinimide (NHS) was purchased from Tokyo Chemical Industry, Japan. Glutamic acid was supplied by Yi Jiang Chemical Co., Ltd, Shanghai, China. All the reagents were of analytical grade and used without further purification. The male New Zealand white rabbits were supplied by the First Affiliated Hospital of Harbin Medical University (Harbin, Heilongjiang Province, China). The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. All animals were handled in accordance with the Chinese National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2 Amination of pristine MWCNTs

To introduce the aromatic amines on the surface of the pMWCNTs, the p-amino benzoic acid, pMWCNTs and poly phosphoric acid were added into a three-neck round-bottom flask equipped with nitrogen inlet pipe, and mechanical stirrer. The reactants were mechanically stirred at 120 °C for 3 h under nitrogen atmosphere to form a homogenous mixture. Then, P2O5 was added to the mixture, and then the mixture was heated for 12 h. The reacted mixture was cooled and diluted with distilled water, and the precipitates were washed with the ammonium chloride solution. Then, the materials were washed with distilled water and then vacuum-filtered through a 0.22 μm millipore polycarbonate membrane. The filtered solid was dried in oven at 50 °C over night. The chemical reaction is schematized in Fig. 1(a).

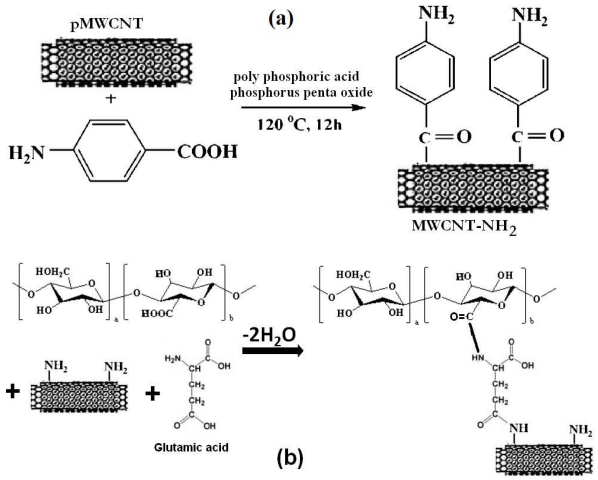

Fig. 1 Scheme of Reactions: Amination of pristine MWCNTs(a), Preparation of MWCNT-NH_2s/ORC composites(b).

2.3 Preparation of ORC

Prior to oxidation, regenerated cellulose filaments were oxidized by using the method as described in our previous works. Briefly, first NO2 was dissolved into CCl4 to prepare 20% (wt) NO2/CCl4 oxidant solution, then added regenerated cellulose into a round bottomed flask containing mentioned oxidant in a proportion of 1:42.6 (g/ml) (fiber: oxidant). Stirred constantly, kept the reaction temperature at 19.5 ◦C and oxidation duration was 40 h. After the reaction, washed the product thrice with CCl4, and then washed the product thrice with the aqueous solution containing 50% (v/v) ethanol followed by washing the product thrice with 100% ethanol. Finally, ORC was frozen-dried at −50 ◦ C in vacuum for 48 h[18].

2.4 Synthesis of nanocomposites

Various amounts of aminated MWCNTs (MWCNT-NH_2s) were suspended in 50 ml acetic acid, and sonicated for 10 min to find a homogenous suspension. Then, EDC, NHS and Glutamic acid was added to the suspension and stirred using magnetic stirrer for 10 min at room temperature. Finally, 100 mg ORC was added to the suspension at room temperature and stirred for 12 h. Finally the ORC was fished out and washed with deionizer water four times to remove ungrafted MWCNT-NH_2s from the surface of ORC. The prepared MWCNT-NH_2s/ORC nanocomposie was dried in oven at 40 °C over night. According to the amounts of MWCNT-NH_2s in suspension, (10, 20, 30 and 40 mg) the prepared composites named as MWCNT-NH_2s/ORC1, MWCNT-NH_2s/ORC2, MWCNT-NH_2s/ORC3 and MWCNT-NH_2s/ORC4

respectively. The chemical reaction between MWCNT-NH_2s and ORC is schematized in Fig. 1(b).

2.5 Characterization

The SEM images refer to MWCNTs and the fracture surface of composites were acquired using a Hitachi S-4700 field emission system. The fracture surface of tensile test samples after breaking was sputter coated with a thin layer (ca. 3 nm) of Au prior to SEM imaging. A TEM, HITACH model Mic H-7650 was employed at the acceleration voltage of 100 kV to investigate the fine nanostructure of synthesized materials. For TEM sample preparation, specimens were dissolved in ethanol and then were dropped the solution on 200 mesh carbon coated copper grid and dried them at room temperature. The Fourier transform infrared (FT-IR) spectra of the samples were recorded at room temperature at the range of 400-4000 cm^{-1} by Nicolet-Nexus670 spectrophotometer.

The XPS spectra of MWCNTs and ORC were obtained using a PHI 5700 ESCA spectrometer. Non-monochromatic Al(K_α) photons were used for all the measurements. The atomic composition of the sample surfaces was calculated using the high-resolution peak areas for the main core XPS line of each element in conjunction with the empirical sensitivity factors provided by the instrument manufacturer and the application of a Shirley-type background correction. The binding energy of the C(1s) was set at 284.5 eV as the reference for all other peaks.

The thermo gravimetric properties of prepared materials were investigated using a simultaneous thermal analyzer (ZRY-2P) by scanning from room temperature to 600 °C at heating rate of 20 °C*min^{-1} under nitrogen atmosphere to prevent oxidation of samples.

To determine the hydrophilicity of the neat ORC and its composites, the bulk water absorption of the samples was determined to reveal their hydrophilic behavior. To determine the water uptake, specimens were immersed in deionized water at room temperature to obtain the change in water uptake with respect to time. After specified times, the samples were taken out from the flasks, and weighed after removing the excess surface water by blotting with laboratory tissue. Five samples were measured for each type of composites. The percentage of water uptake was calculated using the following equation:

Percentage of water uptake = [(Wwet -Wdry)/Wdry]*100%;

where Wdry and Wwet are the weights of the samples before and after immersion in water, respectively[19].

2.6 Haemostatic evaluation

The Male New Zealand White rabbits（which is 4 months old and around 3.5 kg）were used to evaluate the amount of excess blood that oozed out during the hemostat formation. The neat ORC and MWCNT-NH_2s/ORC were cut into pieces of required size (1.0 cm×1.0 cm). Prior to an abdominal incision, the rabbits were fixed on the surgical cork board and anaesthetized with an intraperitoneal injection of 3% pentobarbital sodium aqueous solution (30mg/kg). The neat ORC and its composite with aminated MWCNTs were respectively applied to the liver wound immediately after the liver was pricked with a needle (the diameter is 2mm, and the pricked depth is 3mm) for five minutes for blood penetration in the samples and then clot formation on the surface of samples. The haemostatic effect of samples and amount of bleeding were recorded with determining the weight of samples before and after stop bleeding.

3. Results and Discussion

3.1 Morphology of MWCNTs and dispersion on ORC fibres

The nanotube dispersions on ORC were examined by SEM as presented in Fig. 2.

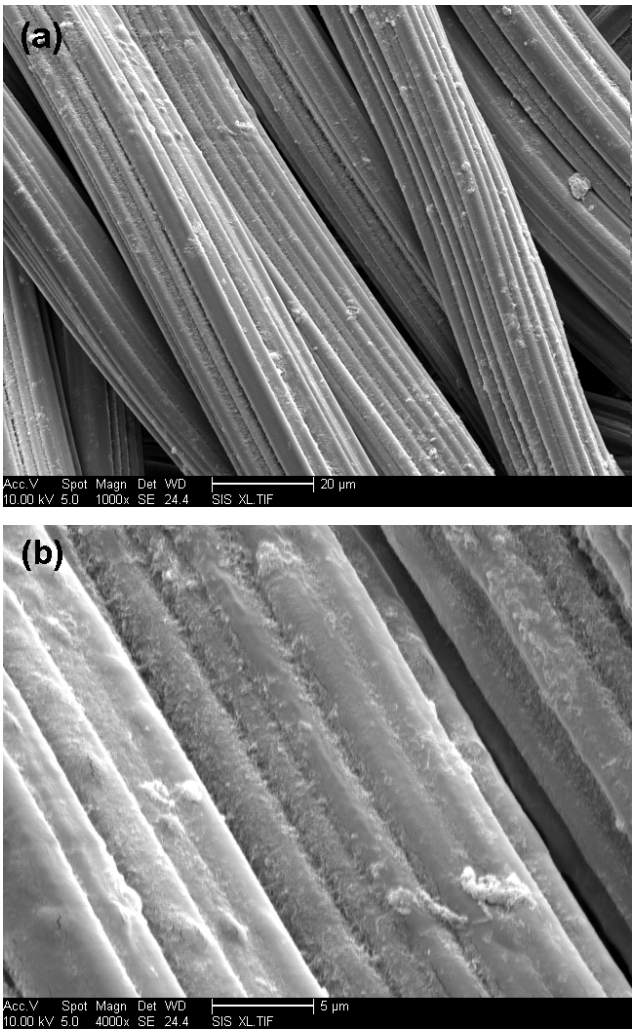

Fig. 2 The SEM micrographs of MWCNT-NH_2s/ORC 3(low Magnification) (a) and MWCNT-NH_2s/ORC3 (high Magnification) (b).

It can be seen that the MWCNT-NH_2s are attached homogenously on the surface of ORC fibers. Also, it can be seen that some of the MWCNT-NH_2s are connected two neighbor ORC fibers to each other. This effect promises increasing the mechanical properties of ORC fibers.

Figure 3 gives the surface morphology of pMWCNTs and MWCNT-NH_2s.

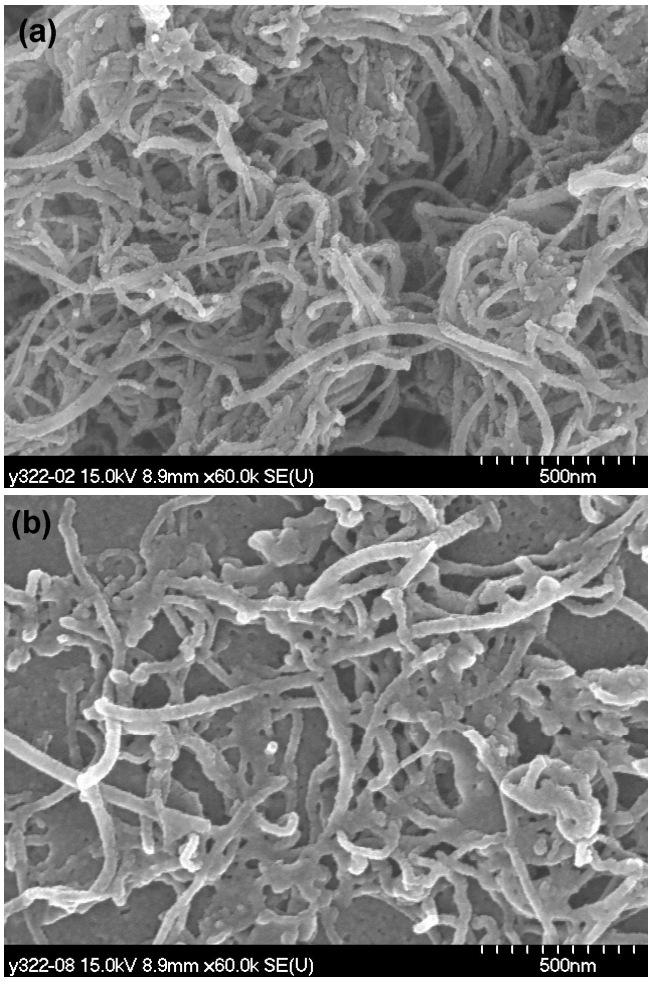

Fig. 3 The SEM micrographs of pMWCNTs (a) and MWCNT-NH_2s (b).

The pMWCNTs are long and varied in diameter. As can be seen, some of the pMWCNTs were entangled to each other. Due to purification and amination, the MWCNT-NH_2s have less entangled points in comparison with pristine MWCNTs. Additionaly, due to the amination process, the diameter of MWCNTs slightly increased during amination, as can be seen in Fig. 3(b).

3.3. Structural characterization of composites

Figure 4 gives the FT-IR spectra of pristine MWCNTs, MWCNT-NH_2s, neat ORC and MWCNT-NH_2s/ORC.

The pristine MWCNTs have some weak peaks between 2980-2840 cm^{-1} corresponds to –CH stretching absorption band. The FT-IR result (–CH stretching) indicates that pMWCNTs contain defects, which may be formed during their manufacture. The FT-IR spectra of MWCNT-NH_2s shows a N-H band at 1235 cm^{-1}, indicating that functional groups were introduced onto the sidewall of MWCNTs. The NH_2 stretch band appears at 3420 cm^{-1}. The scissoring in-plane bending mode of the primary amine NH_2 group at 1645 cm^{-1} is broader than other peaks in this region, such as the carbonyl stretching and aromatic ring modes. A broad band at 758 cm^{-1} is due to the out of plane NH_2 bending mode.

In the FT-IR spectra of neat ORC (Fig. 4(3)), the absorption peak refers to hydroxyl groups is assigned at 3400-3450 cm^{-1}, the typical peak at 2900 cm^{-1} is due to the stretching vibration of -CH_2. The peak at 1083 cm^{-1} are contributed to the stretching vibration of C-O-C. The peak around 1745 cm^{-1} is due to the stretching vibration of C=O, which shows that the oxidation reaction occurs at the hydroxyl groups in regenerated cellulose structure. The FT-IR spectra of MWCNT-NH_2s/ORC in Fig. 4(4) shows that the N–H bending vibration of primary amines is observed in the region 1639 cm^{-1}. Another band attributed to amines is observed around 857 cm^{-1}. This strong, broad band is due to N–H wag and observed only for primary and secondary amines. The C–N stretching vibration of aliphatic amines is observed as medium or weak bands in the region 1230 cm^{-1}. The FT-IR analysis revealed that the amination of pristine MWCNTs and covalent reaction between aminated MWCNTs and ORC has been down successfully.

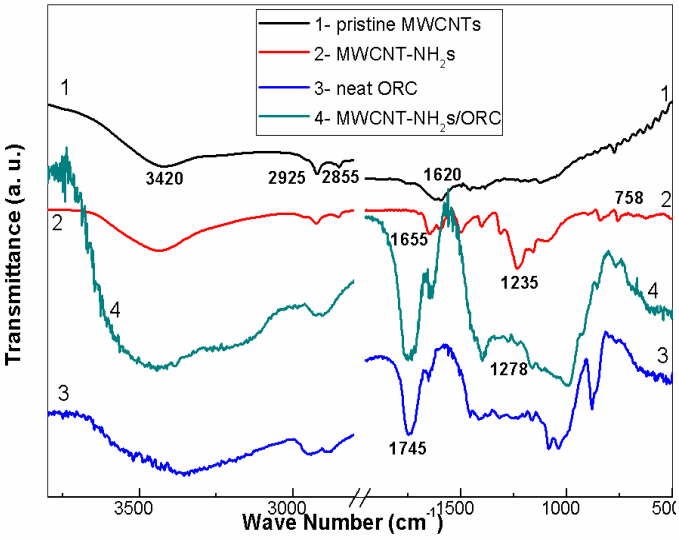

Fig. 4 FT-IR spectra of pristine MWCNTs (1), MWCNT-NH_2s (2) neat ORC (3) and MWCNT-NH_2s/ORC (4).

The XPS analysis can be employed to determine the compositions on the surface of MWCNTs and ORC, the results are introduced in Fig.s 5 & 6. Table 1 shows the XPS semi-quantified atomic concentration for various samples results of the amination of MWCNTs with the relative contents of carbon, Nitrogen and oxygen expressed as atomic percentage (atomic %), as a function of amination and grafting of aminated MWCNTs to the surface of ORC.

Table 1 The XPS analysis of pristine MWCNTs, aminated MWCNTs, neat ORC and MWCNT-NH_2s/ORC.

Materials	element	peak (ev)	Atomic%
pMWCNTs	C 1s	285.3	89.2
	O 1s	533.9	10.8
MWCNT-NH_2s	C 1s	287.3	85.6
	N 1s	401.8	5.2
	O 1s	534.5	9.2
neat ORC	C 1s	289.0	66.0
	O 1s	535.3	31.5
MWCNT-NH_2s/ORC3	C 1s	288.3	70.6
	N 1s	401.9	2.3
	O 1s	535.3	27.1

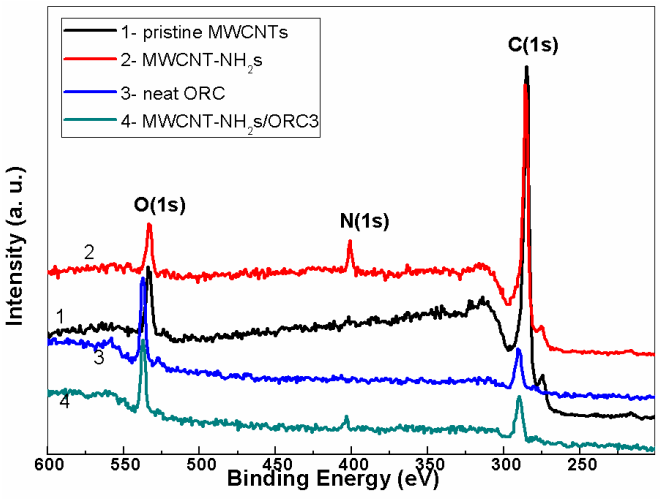

Fig. 5 Wide scan XPS spectra of pristine MWCNTs (1), MWCNT-NH_2s (2), neat ORC (3) and MWCNT-NH_2s/ORC3 (4).

The major peak component at the binding energy (BE) about 285 eV is assigned to the C(1s), the peak component at BE of 532 eV is attributed to O(1s) on the surface of MWCNTs, the peak at the BE of 401.8 eV corresponds to N (1s). The XPS analysis of pristine MWCNTs in Fig. 5(1) shows that the surface of pMWCNTs has some oxygen atoms that refer to the defects and impurities (C-H, C-O, C=O) that formed during manufacturing and storage before using. The peak, commonly related to the π-π* transition levels (free electrons of the graphitic plane) is observed at 291 eV. After amination of pristine MWCNTs, as can be seen in Fig. 5(2), the amount of nitrogen atoms increased gradually that attributes to the amine groups on the surface of aminated MWCNTs. The XPS spectrum of aminated MWCNTs C(1s) peak shows a significant high intensity at a higher binding energy region. This peak is resulted from the amine groups on the tube surfaces.

The peak provides an additional evidence of MWCNT amination. The presence of N-C and N-H bonds on the tube surfaces offers possibilities for tailoring MWCNT surface amination.

Fig. 6(1) shows that pristine MWCNTs contain defects (C-H, C-O, C=O), which are created during their manufacture. The high pure pristine MWCNTs must have just the C-C (sp^2) bonds. Therefore, the C-H, C-O and C=O detected bonds on the sidewall of pristine MWCNTs come from the defects which are created during synthesis of MWCNTs. The peak at 291 eV is commonly related to the π-π* transition levels (free electrons of the graphitic plane).

As can be seen in Fig. 6(2), after amination of pristine MWCNTs, the concentration of nitrogen atoms increase gradually which is attributed to the amine groups that introduced on the surface of MWCNTs. The XPS spectrum of aminated MWCNTs C(1s) peak shows a significant high intensity at a higher binding energy region. This peak is resulted from the amine groups on the tube surfaces. The presence of N-C and N-H bonds on the surface of MWCNTs tube offers possibilities for tailoring MWCNT surface functionalization using amine groups.

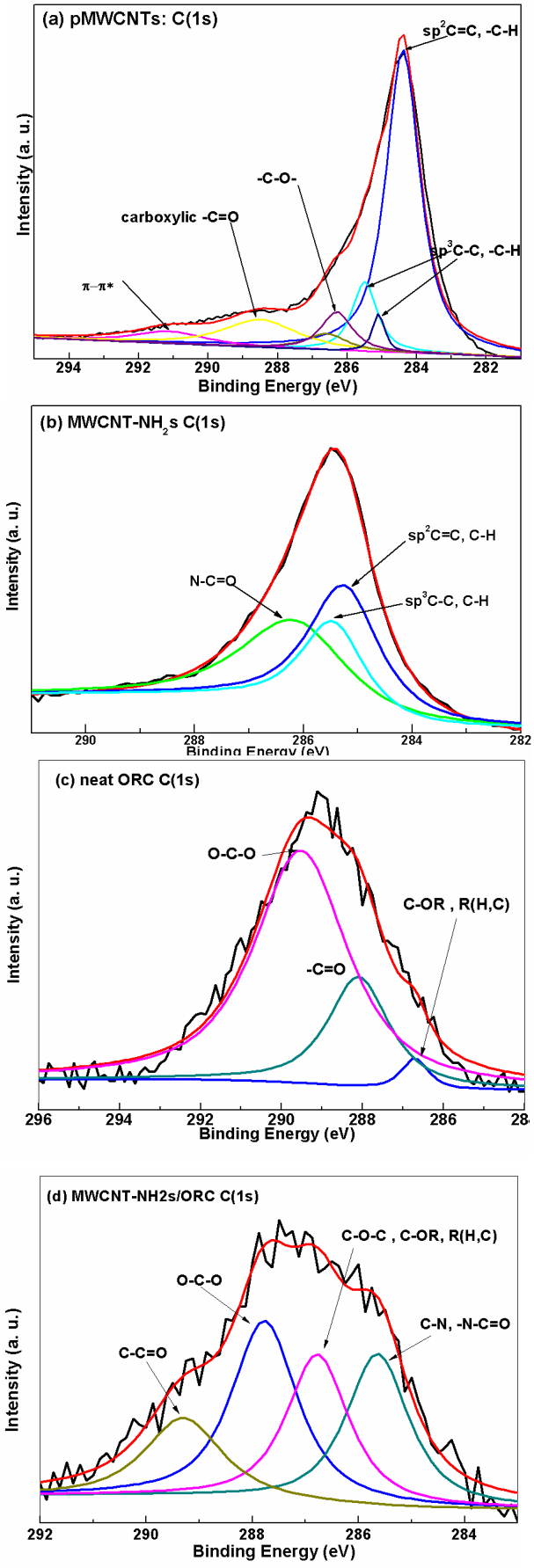

Fig. 6 High resolution XPS analysis of pristine MWCNTs (a), MWCNT-NH_2s (b), neat ORC (c) and MWCNT-NH_2s/ORC (d) at C(1s) region with data deconvolution.

In case of grafting MWCNT-NH_2s to the surface of ORC, detection of –N-C=O and C-N bonds, as can be seen in Fig. 6(4) confirm the covalent bond between two components. These results confirm that the PLLA chains are grafted from the sidewall of MWCNT-NH_2s successfully. Moreover, the disappearance of the π-π* transition levels indicates that the covalent bonds should have been formed between the aminated MWCNTs and ORC macromolecules during the grafting reaction.

These XPS analysis provide the evidence that the amination of pristine MWCNTs and grafting of MWCNT-NH_2s to the surface of ORC are carried out successfully with the mentioned procedure as described above.

3.4. Thermal degradation of MWCNTs and MWCNT-NH_2s/ORC composites.

The thermo gravimetric properties of pristine MWCNTs, aminated MWCNTs, neat ORC and MWCNT-NH_2s/ORC3 composites are presented in Fig. 7.

As can be seen in Fig. 7(1), the Tg curve of pristine MWCNTs shows that they have just 11% weight loss at the temperature range of 100-600 °C, which is contributed to the decomposition of some impurities such as trace of water and amorphous carbon that deposited on the sidewall of pMWCNTs and the diffused materials inside the carbon nanotubes during manufacturing. The TGA curve of MWCNTs-NH_2s shows that the MWCNTs-NH_2s has 25% weight loss from 100 to 600 °C, which is attributed to the decomposition of amine groups that created on the sidewall of MWCNTs during amination and trace of water and ethanol remained after washing[20].

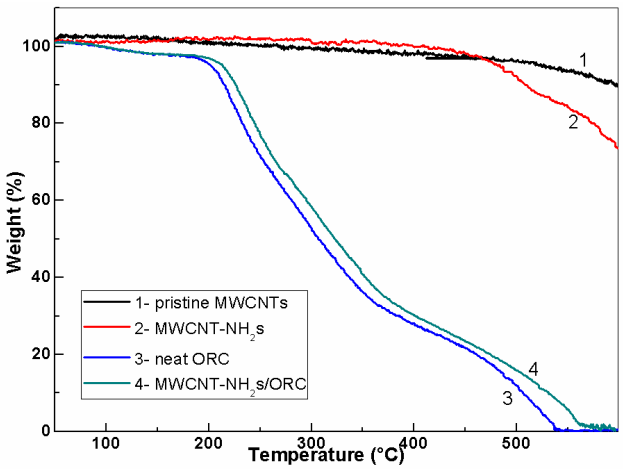

Fig. 7 Thermo gravimetric analysis of pristine MWCNTs (1), MWCNT-NH_2s (2) neat ORC (3) and MWCNT-NH_2s/ORC (4) under nitrogen atmosphere.

The concentration of MWCNTs-NH_2s on the surface of ORC was estimated using TGA of each composite that compared with TGA of neat ORC. For each composite, the concentration of MWCNTs-NH_2s was determined as the residual weight of composites up to 550 °C in comparison with neat ORC after thermal degradation of ORC as presented in table 2.

3.5. Hydrophilicity of MWCNT-NH_2s/ORC

Hydrophilicity is an important characteristic property of hemostatic biomaterials. To determine the hydrophilicity of the composites, the bulk water absorption of the composites was determined to reveal their hydrophilic behaviour. Table represents the results of water absorption for neat ORC and MWCNT-NH_2s/ORC composites.

Table 2 The water uptake of neat ORC and MWCNT-NH_2s/ORC composites in deionized water at room temperature.

Sample	Concentration of MWCNT-NH_2s (wt%)	Water uptake (± 2%)
neat ORC	0	517
MWCNT-NH_2s/ORC1	0.8	529
MWCNT-NH_2s/ORC2	1.4	541
MWCNT-NH_2s/ORC3	2.5	558
MWCNT-NH_2s/ORC4	2.7	560

It can be seen that the MWCNT-NH_2s increased the water uptake of ORC. Fig. 8 represents the schematic representation of water uptake for neat ORC and MWCNT-NH_2s/ORC.

Schematic representation of water uptake for neat ORC is compared with MWCNT-NH_2s/ORC as shown in Fig. 8. Due to amine group on the surface of grafted MWCNTs and also, carboxyl group on the attached glutamic acid the water uptake of composites is extensively higher than that of neat ORC.

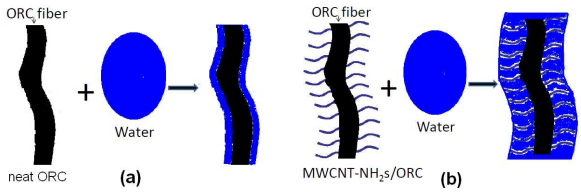

Fig. 8 Scheme for water uptake of neat ORC (a) and MWCNT-NH_2s/ORC (b).

3.6 Haemostatic evaluation

The bleeding from the rabbit liver is affected by a number of factors such as blood pressure and size of the liver. To minimize the effect of the experimental error, 3 rabbits for each sample were carried out. The results of haemostatic evaluation were summarized in Fig. 9. The experiments show that both of neat ORC and MWCNTs-NH_2s/ORC3 stop bleeding by blood uptake and coagulation of absorbed blood on the surface of samples. The average blood uptake of neat ORC was 439.7% and that of MWCNTs-NH_2s/ORC3 was 355.1%. It means that the application of MWCNTs-NH_2s/ORC3 decreases the blood loosing from the injured site of liver. In addition, the aminated MWCNTs increases the rate of stop bleeding.

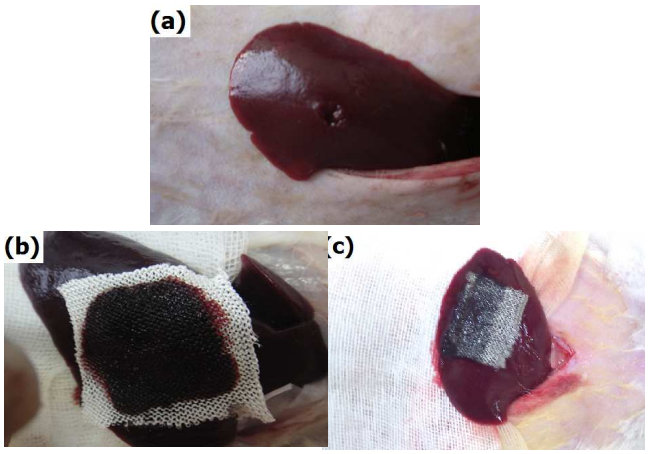

Fig. 5 Photographs of injured site of rabbit liver (a) and hemostatic evaluation of neat ORC (b) and hemostatic evaluation MWCNTs-NH_2S/ORC3.

Conclusions

In this research, a novel technique for covalent conjugation of MWCNTs to ORC is introduced. At first, the pristine MWCNTs were aminated without shortening of MWCNTs. Then, the aminated MWCNTs were covalently grafted to the surface of neat ORC using glutamic acid as cross linking bridge. The FT-IR and XPS analysis confirms the covalent reaction between aminated MWCNTs and ORC surface. The water uptake and the haemostatic effect of ORC increased with introducing a small percentage of aminated MWCNTs. This research suggests that the hemostatic properties of ORC can be modified by introducing a small percentage of aminated MWCNTs. The combination of ORC and CNTs opens in fact a new perspective in the self assembly of nanomaterials and nanodevices for biomedical applications especially hemostatic effect.

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Notes and references

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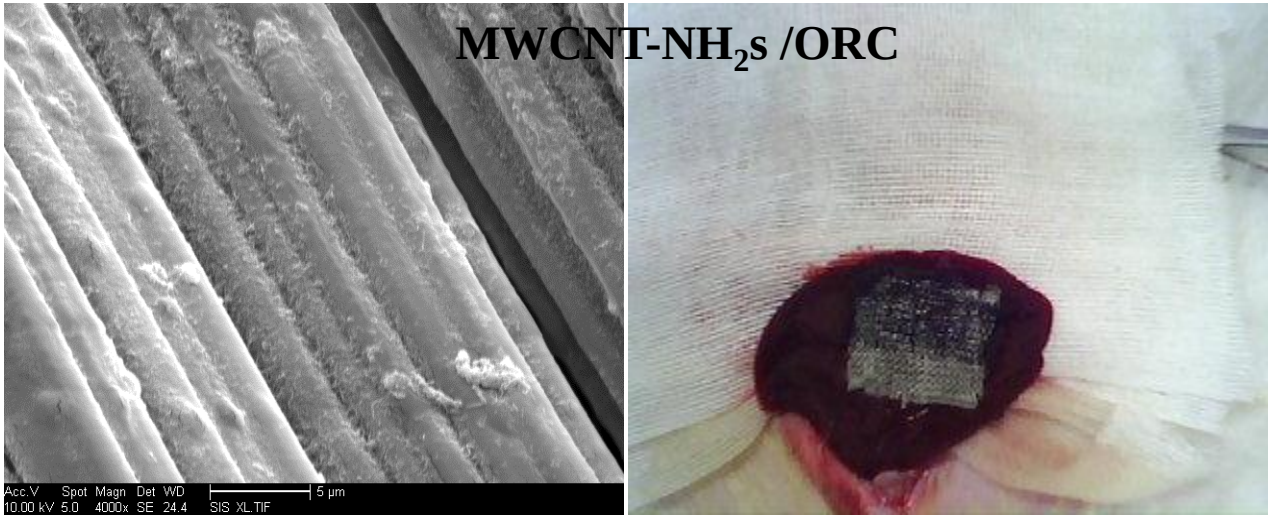

The hemostatic effect of oxidized regenerated cellulose (ORC) was enhanced using a minated MWCNTs which covalently grafted to the surface of ORC of using glutamic acid as crosslinking bridge. With small percentage of aminated MWCNTs, the water u ptake and hemostatic effect of ORC increased gradually.