Improving Antibacterial Activity of Methicillin By Conjugation To Functionalized Single-Wall Carbon Nanotubes Against *S. Aureus*

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Abstract

Methicillin-resistant *Staphylococcus aureus* is a problem in both healthcare institutions and community settings. This is due to its multi-drug-resistant challenges. Recent advancement in nanotechnology has expanded our ability to design and construct nanomaterials to treat bacterial infections. Carbon nanotubes are one of these nanomaterials. Herein, twenty isolates from hospitals were identified as *S. aureus* based on morphology, Gram stain, catalase test, coagulase test, and mannitol salt agar fermentation. Isolates were subjected to bio-typing, the methicillin resistance was characterized using the disk diffusion method and PCR of the *mecA* gene. Then, the single-walled carbon nanotubes (SWCNTs) were functionalized and conjugated to methicillin. The prepared nano-antibiotics were characterized using FTIR and SEM analysis. The characterization of the nano-antibiotics confirmed the successful functionalization of the SWCNTs and the addition of carboxyl groups. Interestingly, the results of the antibacterial activity indicated that the functionalized methicillin-loaded SWCNTs have a significant increase in the antibacterial activity against the *S. aureus* in comparison to the methicillin-free drug.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a public health concern everywhere. It is the cause of morbidity and mortality compared to other pathogenic bacteria. The elevated colonization rates lead to the rising rate of infection in the society which, leads to a significant increase in treatment cost (Anderson; Mitchell, 2000; Tomasz, 1994).

S. aureus is well-known for developing antibiotic resistance. Infections caused by antibiotic-resistant strains frequently occur in epidemic waves triggered by a single or a few successful clones. During these outbreaks, MRSA plays a key role (Blot, Vandewoude, & Colardyn, 1998). Because the shelf life of antibiotics is limited, it will not be possible to trustfully use the available antibiotics in the treatment of bacterial infections. That's why the number of FDA-approved antibiotics has been steadily declining over the past few decades.

As a result, there is a great need to develop novel antimicrobial medicines that can effectively treat antibiotic-resistant bacteria. One promising method is to use suitable nanomaterials for the selective treatment of bacterial infections. Materials with at least one dimension in the nanometer scale range (1 to 100 nanometers) are called nanomaterials. These nanomaterials have a high surface area to volume ratios and unique chemo-physical properties, all of which contribute to their antibacterial efficacy (Cheon & Lee, 2008; Gao, Gu, & Xu, 2009; Hajipour et al., 2012; Taubes, 2008; Taylor & Webster, 2011). For example, ZnO can inhibit *S. aureus*, and Ag exhibits concentration-dependent antimicrobial activity against *E. coli* (Ramalingam, Parandhaman, & Das, 2016).

Various proposed antibacterial mechanisms involved damaging the bacterial cell wall/membrane, synthesis oxidative stress induction, interruption of energy transduction, metal ion release, and inhibition of enzyme activity (Huhand & Kwon, 2011; Seil & Webster, 2012).

Among nanotechnology-derived materials, carbon nanotubes (CNTs) have stimulated great interest in biomedical applications because of their unique mechanical, electrical, thermal, and spectroscopic properties. It is important to note that recent studies showed that the functionalization of CNTs with different approaches increases biocompatibility and decreases toxicity (Eatemadi et al.; Liu et al., 2007).

New findings of *S. aureus* isolates with resistance to a wide range of antibiotics create a challenging problem where effective bactericidal antibiotics may no longer be readily available against this organism. In the present study. We tried to examine the efficacy of antibiotic-loaded single-wall carbon nanotubes (SWCNTs) to reach new antibacterial agents toward infectious diseases.

Materials And Methods

Clinical Strains

Twenty isolates (blood, cerebral spinal fluid, urine, wound swab, urethral swab, and sputum) of staphylococci were collected from subjects attending various hospitals in Tehran (Iran) after obtaining their informed consent. Methicillin-resistant *S. aureus* (MRSA) ATCC33591 from Pasteur Institute of Iran was used as control.

Pathogen Identification

An isolated pathogen is identified using biochemical, enzymatic, or molecular tests. All primary cultures were subcultured on Mannitol Salt Agar (ALPHA) and incubated using an incubator (Gallenkomp, England) at 37°C for 18–24 h. Yellow colonies were selected. Samples were further identified by routine laboratory procedures, including microscopic morphology and biochemical tests including Gram staining and catalase (Harrigan, 1998).

DNase agar test achieved by incubating the isolates at 37°C for 24 hours on DNase agar (Thermo Fisher Scientific, Basingstoke, UK.), and overflow the plate with 1 N HCl. Merely potent DNase activities, with a large clearing zone around the spotted colony, are positive for DNase.

Coagulase tube tests are also effective in diagnosing *staphylococci*. Pure colonies of gram-positive and catalase-positive *staphylococci* are detectable after *enterococci* use citrate (Kateete et al., 2010). Positive control tubes containing citrated plasma and *S. aureus* strain producing coagulase were incubated for 4 hours at 35°C and then examined for clot formation. If not coagulated, the tubes should be incubated for another 18 hours at room temperature (Kateete et al., 2010). They were then examined without stirring so that the clots not disrupt.

To evaluate the hemolysis of isolates, after overnight incubation at 37°C on sheep blood agar prepared with 15 ml of 5 % sheep blood in Trypticase soy agar (Becton Dickinson) as an overlay on 10 ml of blood agar base. Hemolysis recorded as α-hemolysis, β-hemolysis, double hemolysis (α + β), and negative (no hemolysis)(Salih, Alrabadi, Thalij, & Hussien, 2016).

Molecular assay

Bacterial DNA was isolated from all pure staphylococcal strains using a PCR kit (Bioneer, Korea). PCR-amplification reaction for the *mecA* gene used as a baseline test performed according to the Kit instruction with the following primers; F: 5'-GAGATAGGCATCGTTCCAAAGAATG-3' and R: 5'-GTTTTAATTCTTCAGAGTTAATG-3'. Then amplified PCR product of 290 base pairs was visualized on 2% agarose gel. The reference gene *mecA* from *S. aureus* was used as a positive control, while NCTC 6571 was applied as negative control (Shittu, Lin, Morrison, & Kolawole, 2006).

Antibiotic susceptibility test

Isolated pathogens are tested independently to determine the potent of antimicrobials to inhibit their growth. The qualitative results of the agar diffusion method help to determine antibiotic susceptibility (Bauer & Kirby; Jorgensen & Ferraro, 1998). A pure colony of each isolate was inoculated and incubated in a tube containing 2 ml of liquid culture medium (Oxoid, UK). The overnight physiological culture was diluted with sterile distilled water to bring the turbidity to 0.5 McFarland standard. A sterilized swab stick was dipped into the adjusted suspension and streaked over the surface of already prepared Mueller-Hinton Agar (Merck) plates. The antibiotic discs: cephalexin, oxacillin, meropenem, vancomycin, cefoxitin, and methicillin (Abtek), were applied onto the inoculated plate. Followed by incubation on 18–24 h at 37°C, the diameter of the inhibitory zones was measured and then interpreted according to the CLSI criteria (2015)(Clinical & Institute, 2017). Besides, to estimate the minimum inhibitory concentrations (MICs) for MRSA isolates, E-test was used according to manufacturers' instructions.

Chemical Functionalization of SWCNTs

The SWCNTs become functionalized by the intense acid solution. The acid solution containing H2SO4: HNO3 with a ratio of 3:2, with acid concentrations of 98% and 65%, respectively, was added to a flask comprising the SWCNTs. After The mixture's incubation at 140°C for 4 hours, it was diluted with distilled water and stirred for 6 hours. The SWCNTs were washed thoroughly with distilled water until pH reached 7 and filtered by membrane filters (0.2μm). After the filtration process, the

functionalized SWCNTs (f-SWCNT) were left in a dry oven for 24 hours at 60°C. The functionalized SWCNTs were then characterized by FTIR spectroscopy (Perkin Elmer Spectrum II) and using Scanning Electron Microscopy (Porto, Silva, Santos, & de Oliveira, 2018; Tsai, Kuo, Chiu, & Wu, 2013).

Attachment of methicillin on f-SWCNTs

To the mixture of methicillin (25 mg), f-SWCNTs (25 mg) were added to 10 mL of distilled water. The reaction run overnight at room temperature. The reaction mixture was sonicated for 5 min and centrifuged at 6000 rpm for 10 min. The pellet was washed with ultrapure water, and the products dried under a vacuum (Tsai et al., 2013; Williams, Nayeem, Dolash, & Sooter, 2014).

Broth microdilution method

This approach was applied to find out the MIC of methicillin lonely and in combination with SWCNTs. In summary, methicillin dissolved in dimethyl sulfoxide (DMSO) for a concentration of 8 µg/ml. Though, again the concentration of 8 µg/ml per 146 µg/ml of f-SWCNTs was provided by distilled water. Then, using Muller Hinton broth (Merck), prepared ten times twofold serial dilution. Detecting the DMSO antibacterial activity, it was also diluted in a series (twofold) with Muller Hinton broth to reach concentrations of 0.1–50%. Then, the negative control received the final bacterial concentrations of 5×10^5 CFU/ml in each well. The overnight grown bacterial isolates poured into all wells in duplicate. After bacterial inoculation, the plates were incubated for 18 hours at 37°C. The MIC was detected by plate absorption at 630 nm using a Microplate reader (Bio-Rad, USA), with Muller Hinton broth as the blank (Assali, Zaid, Abdallah, Almasri, & Khayyat, 2017).

Results

Sensitivity and specificity of the tests

S. aureus identification employs the commonly accepted methods of catalase, coagulase, DNase, and MSA. This study was performed to determine how often yellow colonies were detected on the MSA plate definitively identified as *S. aureus*. Colonies in nutrient agar showed circular, convex, shiny, opaque, smooth forms and colonies with slightly golden-yellow pigment. In blood agar test, *S. aureus* colonies were β-hemolytic, white, or cream-colored. Tested *S. aureus* isolates showed a vivid DNase activity. Accordingly, the Gram-staining method certified the gram-positive cocci in clusters. The repeated catalase test was positive with 3% H_2O_2 on a glass slide.

Coagulase testing is the most reliable method for detecting *S. aureus* (Bauer & Kirby). In this study, coagulase production was detected using a coagulase smear test. Coagulase slide detects coagulase, reacts directly with fibrinogen in plasma and causes rapid cell agglutination (Louie, Majury, Goodfellow, Louie, & Simor, 2001). The coagulase test was not sensitive after 4 hours, but only after 24 hours of incubation represented correct results. The results are shown in Table 1.

Table 1
The biochemical test for the identification of isolated *S. aureus*.

Test	Results
Grams Staining	+
Catalase	+
Coagulase	+
DNase	+
Mannitol Fermentation	+
Blood Agar Hemolysis	+

Status of methicillin-resistant genes and antibiogram

The results obtained by E-test reflected that all the methicillin-resistant *S. aureus* isolates were strongly resistant to methicillin with minimum inhibitory concentrations varying from 4 to 256 μg/ml, as illustrated in Fig. 1. The organisms that showed resistance toward methicillin are called MRSA.

The results achieved via E-test displayed that all the MRSA isolates were fully resistant to methicillin with the MIC varying from 4 to 256 μg/ml, as depicted in Fig. 1. The isolate susceptibility to antibiotics was determined using the disk diffusion method according to CLSI criteria (Bauer & Kirby). The strain susceptibility exhibited being sensitive to vancomycin and resistance to meropenem, cefoxitin, and intermediate state toward cephalexin and oxacillin (Table 2). The DNA extraction was carried out to detect the level of sensitivity pattern between the antibiotic-resistant strains of MRSA and determining the presence of the *mecA* gene by PCR (Fig. 2). All subjects of MRSA isolates were *mecA* positive and produced the band of 290 bp specific for the *mecA* gene.

Table 2
In vitro antibiotic sensitivity pattern of *S. aureus*.

Antibiotics	Diameter of zones (mm)																			
Strains No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Methicillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Vancomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Oxacillin	R	R	R	S	S	S	R	R	S	R	S	R	S	R	R	S	R	S	R	R
Meropenem	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefoxitin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cephalexin	R	R	R	R	R	R	I	I	R	I	I	R	I	R	R	R	R	I	R	I

R = Resistant, S = Sensitive, I = Intermediate

Scanning Electron Microscopy

Surface morphology was observed for f-SWCNTs and antibiotic-loaded SWCNT through a scanning electron microscope. Acid treatment of SWCNTs using the acid mixture makes the surface roughened, such as in (Fig. 3a) and (Fig. 3c). No impurity was detected in acid-treated SWCNTs. The roughness of the side walls is attributed to defect sites with carboxyl groups on the surface. Antibiotics on the surface of SWCNT, as shown in (Fig. 3b).

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy was performed to verify the existence of carboxyl groups and methicillin. Fourier transform infrared spectra were recorded from 1000 cm-1 to 3500 cm-1, and the specter of the SWCNT, f-SWCNT, and methicillin-SWCNT, shown in Fig. 4. The f-SWCNT and antibiotic-SWCNT show the peaks for the carboxyl group at 1661cm-1 (range 1740 – 1700 cm-1) and the hydroxyl group at 3432 cm-1 (range 3300 – 2500 cm-1) which were absent in SWCNT, thus proving that the SWCNTs were now carboxyl functionalized. In addition, more peaks for the f-SWCNT and antibiotic-SWCNT confirmed the presence of more functional groups.

Antimicrobial Activity of methicillin loaded SWCNTs

SWCNTs functionalized by treating with methicillin to make a novel stable nano-antibiotic. As methicillin was loaded successfully on the surface of the SWCNTs, the antibacterial activity of the new nano-antibiotic was determined. The MIC offered by the methicillin and methicillin-loaded SWCNTs, listed in Table 3 for control and each isolate. The obtained values of MIC for methicillin are higher (at least two-fold) than those observed with methicillin-loaded SWCNTs. However, the blank DMSO did not induce any noticeable antimicrobial activity.

Table 3
In vitro MIC results for methicillin alone and methicillin-SWCNTs when tested against *S. aureus* isolates using broth microdilution method.

Antibacterial elements	Antibiotic (µg/ml)	CNT + Antibiotic (µg/ml)
Control	1024	256
1	512	256
2	1024	128
3	512	64
4	256	128
5	256	128
6	128	32
7	256	16
8	256	32
9	128	32
10	256	64
11	2048	64
12	2018	64
13	512	128
14	256	64
15	512	64
16	512	512
17	2048	512
18	2048	512
19	512	128
20	1024	256

Discussion

The isolation of *S. aureus* from various clinical samples indicates its wide distribution. This distribution could be evidence of *S. aureus* versatility and cause of most nosocomial-associated infections. All of the biochemical tests have confirmed that the strain is *S. aureus*. Since many diagnostic laboratories have not routinely done the catalase test, the true incidence of catalase is not identifiable. While Gram staining, colonial morphology, the coagulase test, and other biochemical tests are more favorable for *S. aureus* identification.

Therefore, the present study has attempted to explore molecular detection of the *mecA* gene from infected patients, which was positive for all strains. In a survey conducted by Louis et al., the PCR test was also conducted on 200 staphylococci to determine the *mecA* gene as the standard gold for *S. aureus* identification (Louie et al., 2001).

Disk diffusion test is now an accepted method for the *S. aureus* resistance detection by an increasing number of reference resistance groups, considering CLSI. All antibiotics used in this study did not exhibit significant effects on the *S. aureus* strains except vancomycin. This observation was contrary to the research of Zeng et al. (Hawkins & Byrne, 2015), who used a

combination of antibiotics (methicillin, vancomycin). Recent studies have also shown that antibiotics susceptibility, controlled by bacterial metabolism, dictated this phenotype (Corona & Martinez, 2013). This regulation makes bacteria either more resistant or more susceptible to antibiotics. However, timed bacteria become momentarily resistant to antibiotics even in the absence of a genetic change (Vallino, 2003).

The antibacterial activity of methicillin-loaded f-SWCNTs was higher than that of methicillin alone by 16 folds against *S. aureus*, as shown in Table 2. The improvement is most likely due to the aggregation of bacteria with the SWCNTs, which increases the exposure of bacteria to methicillin. Consequently, it increased the concentration of antibiotics that entered into bacteria. In addition, this nano-antibiotic will impair the efflux pump mechanism used by bacteria as the mechanism of resistance to antibiotics, as reported by Yang et al., who examined the effect of SWCNTs length on their antimicrobial activity. The longer the SWCNTs were, the stronger was the antimicrobial activity due to their improved aggregation with bacterial cells. (Arias & Yang, 2009; Yang, Mamouni, Tang, & Yang, 2010). The prevalence of MRSA and its resistance pattern are signaling that healthcare workers and the general public are at risk. The desired nano-antibiotic was synthesized successfully to achieve a highly stable nano-antibiotic linked with methicillin. The f-SWCNTs showed good physical stability in water. Moreover, our nano-antibiotic was characterized by various analytical techniques such as SEM and FTIR. Most importantly, the antibacterial activity was improved significantly compared to the methicillin alone against *S. aureus*, as confirmed by the broth dilution method.

Abbreviations

CLSI: **Clinical and Laboratory Standards Institute**

f-SWCNTs : functionalized Single-Walled Carbon Nanotubes

FTIR: Fourier Transform Infrared

SEM: Scanning Electron Microscope

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests. There are no other persons who satisfied the criteria for authorship.

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*Authors' Contributions

Rouhani Nejad, Alami and Saeedi reviewed literature, outlined, wrote the manuscript. Khavari nejad edited the manuscript prepared figures. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Figures

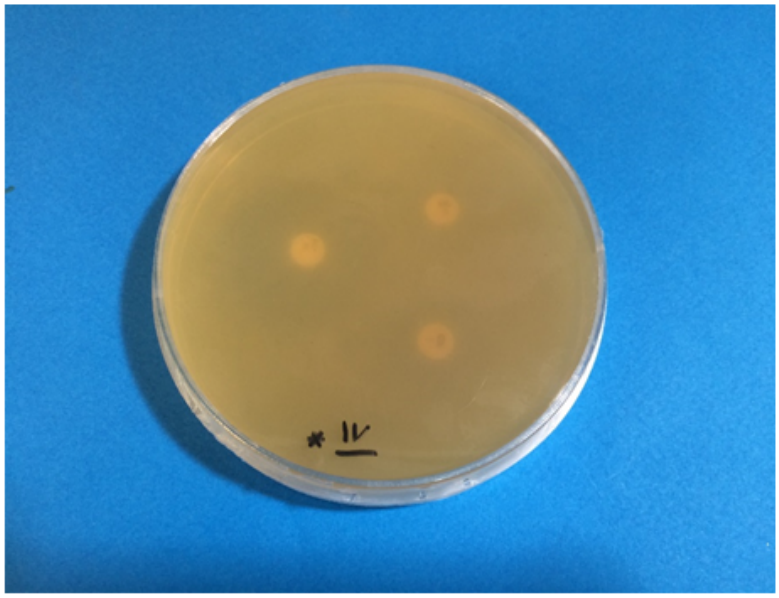

Figure 1

S. aureus showing methicillin-resistant.

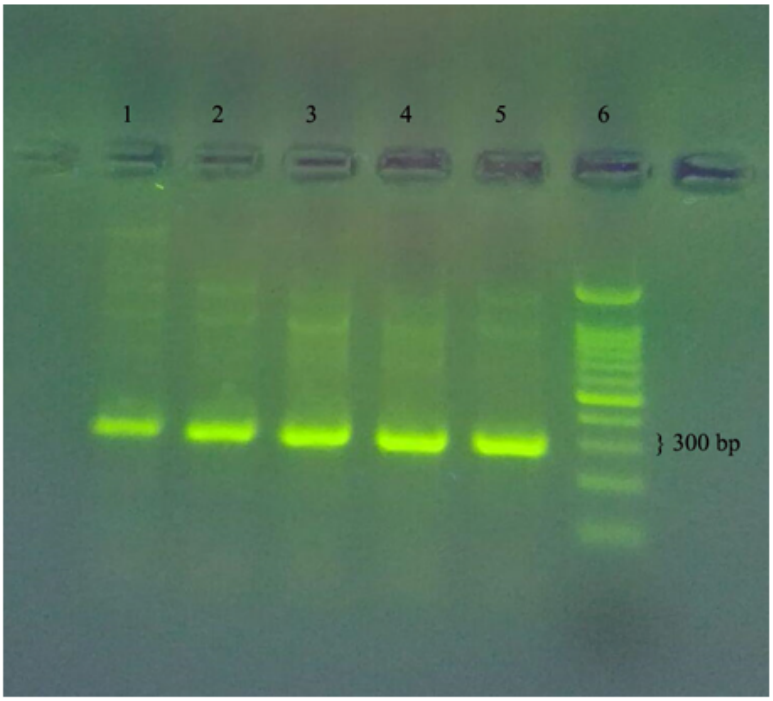

Figure 2

Polymerase chain reaction amplification of mecA gene.

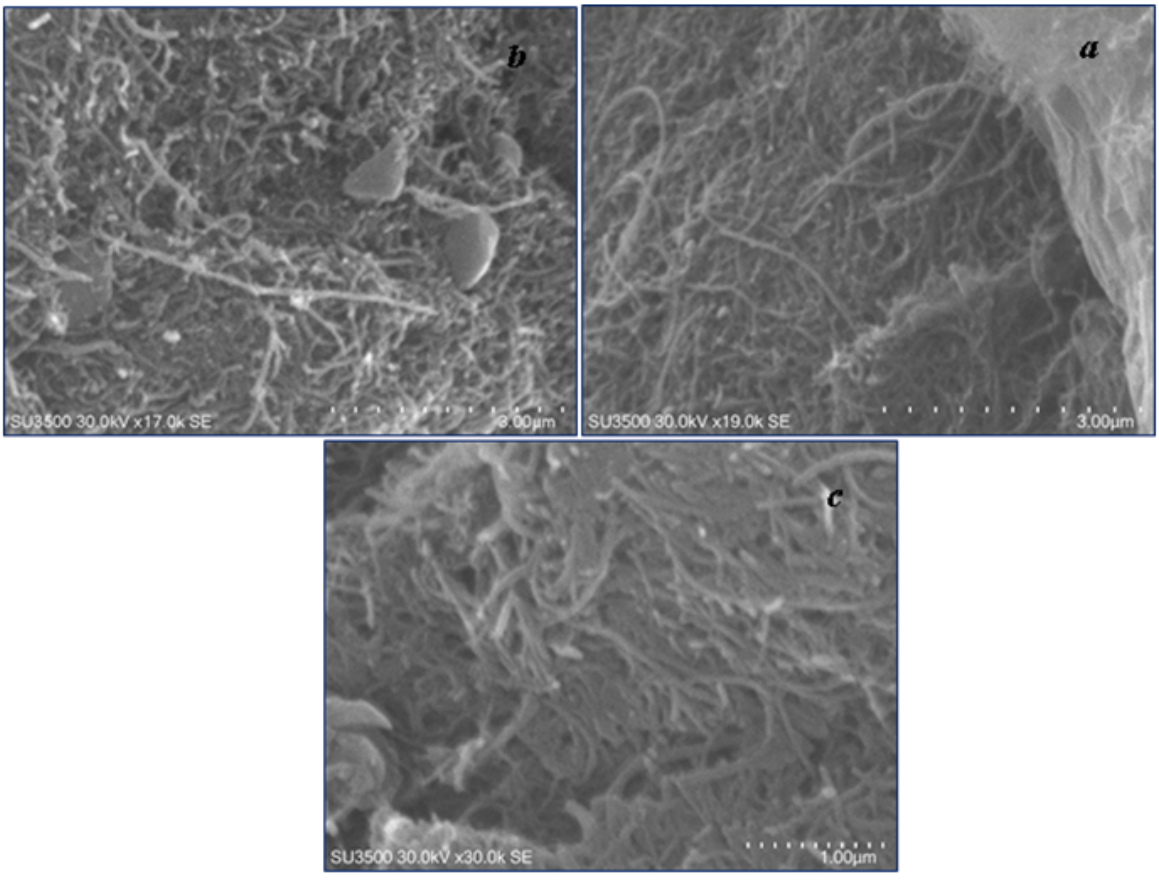

Figure 3

Surface morphology examined by scanning electron microscope. (a) f-SWCNTs, (b) methicillin- SWCNT, (c) f-SWCNT at a larger scale.

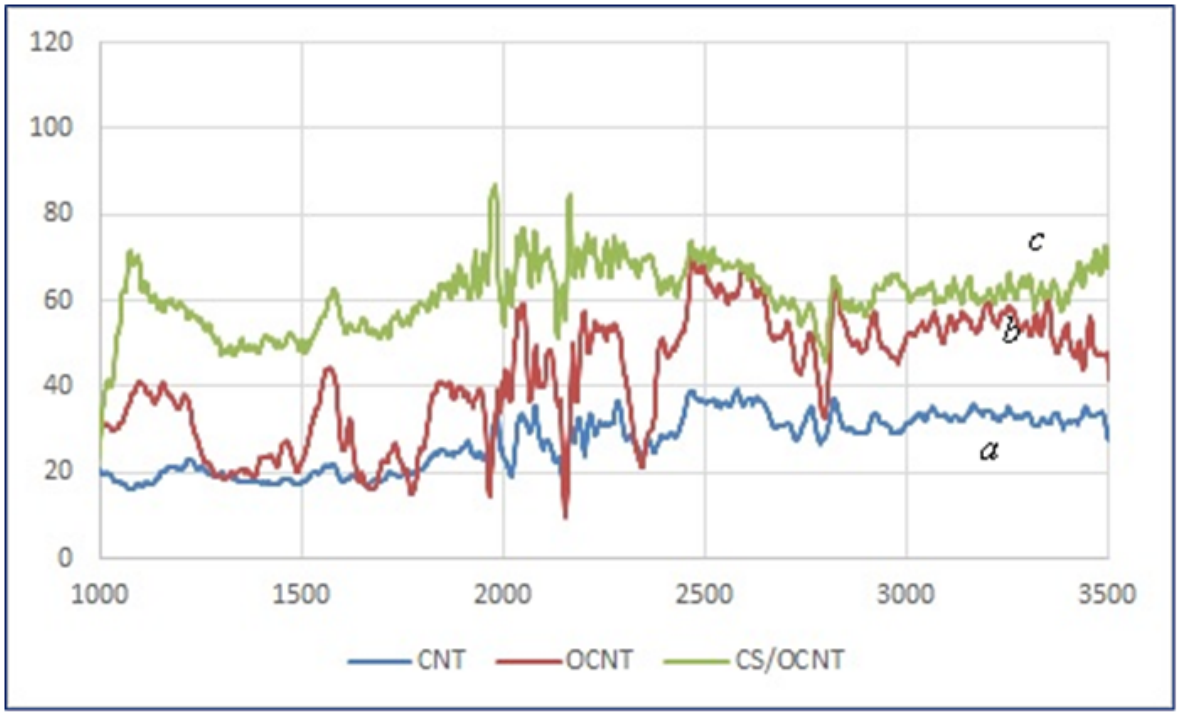

Figure 4

Fourier transform infrared spectroscopic spectra. (a) SWCNTs, (b) functionalized SWCNTs, (c) Methicillin-SWCNT