An-Najah National University

Faculty of Graduate Studies

"Multi-functionalization of single walled carbon nanotubes for antibacterial activity"

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Dedication

To my wonderful father and soulful mother, who have been a great source of inspiration and support me throughout my life and raised me to my dreams.

To my brothers and sisters (Mohammad, Mahmoud, Ahmad, Abeer, Aseel, Reem, Hadeel, Qusai) who have supported me.

To grandparents and big family

To my dear friends

I dedicate this work

Acknowledgment

In the name of Allah, praise Allah for His strength in performing this thesis. Particular appreciation to my supervisor's Dr. Mohyeddin Assali and Dr. Motasem Almasri for their support, supervision and their invaluable assistance of constructive suggestions during the experimental works had contributed to the success of this thesis.

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My parents, and also to my sisters, brothers and my friends deserve warm thanks for the encouragement and endless love, their kindness means a lot to me. Thank you very much.

Kholoud isam aboalrob

الاقرار

أنا الموقعة أدناه مقدمة الرسالة التي تحمل العنوان:

Multi-functionalization of single walled carbon nanotubes for antibacterial activity

أقر بأن ما اشتملت عليه الرسالة هو نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه حيثما ورد، وأن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أي درجة أو لقب علمي أو بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provide in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name **اسم الطالبة:**

Signature: **التوقيع:**

Date: **التاريخ:**

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Symbol	Abbreviation
µL	Microliter
Anhydrous $CuSO_4$	Anhydrous copper sulfate
AUC	Area under the curve
Boc_2O	Di-tert-butyl-dicarbonate
B-PEIs	Branch- polyethelyneimines
C_3H_8O	Isopropyl alcohol
$CDCl_3$	Deuterochloroform
CFU	Colony forming unit
CH_3CN	Acetonitrile
$CHCl_3$	Chloroform
CIP	Ciprofloxacin
Cipro	Ciprofloxacin
CL	Clearence
CLA	Clarithromycin
CLSI	Clinical and laboratory institute
CNTs	Carbon nanotubes
Co	Cobalt
DCM	Dichloromethane
DDS	Drug delivery systems
DIPEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DNA	Deoxyribonucleic acid
EDC	Ethylcarbodiimide hydrochloride
Et_3N	Trimethylamine
EtOAc	Ethylacetate
Fe	Iron
f-SWCNTs	Functionalized single-walled Carbon Nanotube
HCl	Hydrochloride
Hr	Hour or Hours
IR Spectroscopy	Infrared Spectroscopy
Iv	intravenous
K_2CO_3	Potassium carbonate
Log P	partition coefficient
L-PEIs	Linear- polyethelyneimines
MeOH	Methanol
$MgSO_4$	Magnesium sulfate
MHB	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
Min	Minute

MW	Molecular weight
MWCNTs	Multi-walled Carbon Nanotubes
Na_2CO_3	Sodium carbonate
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
NaOH	Sodium Hydroxide
NH_4Cl	Ammonium Chloride
NH_4OH	Ammonia
Ni	Nickel
Nm	Nano meter
NMR	Nuclear Magnetic Resonance
^oC	Celsius
o-DCB	Ortho-Dichlorobenzene
P. aeruginosa	*Pseudomonas aeruginosa*
PAMAM	Poly amidoamine
PBS	Phosphate buffer saline
Pd/C	Palladium on carbon
PEI	Polyethelyneimine
PH	Power of hydrogen
PHT	Oxaheptadecanethiol
PIBCA	Polyisobutylcyanoacrylate
PKa	Acid dissociation constant
PLL	Poly-L-Lysine
p-SWCNTs	Pristine single-walled Carbon Nanotubes
QPEI	Quaternary polyethelyneimine
RBCs	Red blood cells
RBF	Round bottom flask
Rpm	Round per minutes
RT	Room temperature
S. aureus	*Staphylococcus aureus*
S. Typimurium	*Salmonella Typimurium*
S.epidermidis	*Staphylococcus epidermidis*
SWCNT	Single-walled Carbon Nanotube
$T_{1/2}$	Half time
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEG	Tetraethylene glycol
TEM	Transmission electron microscope
TFA	Trifluoroacetic acid
TGA	Thermogravimetric analysis

TLC	Thin layer chromatography
TsCl	Tosyl chloride
UTI	Urinary tract infection
UV-Vis	Ultraviolet-Visible
Vd	Volume distribution
VdW	Van der waals
ZnO	Zinc Oxide
λ_{max}	Lambda max

Multi functionalization of single-walled carbon nanotubes for antibacterial activity

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Abstract

Infectious diseases are considered the greatest problem that people suffer and challenge worldwide. The wide use of antibiotic has led to increasing resistance to these drugs. Accordingly, many researchers are eager to develop new drug delivery systems that may result in decreasing the side effects and the effective dose of the drug. One of the occasional drug delivery systems in this field is based on carbon nanotubes technology. Furthermore, cationic compounds as hopeful candidates for the reduced potential for resistance development.

The aim of this work is to develop a new nano-antibacterial system based on the multi-functionalization of single-walled Carbon Nanotubes (SWCNTs) using covalent functionalization of SWCNTs-COOH with ciprofloxacin and multiamine linker combined noncovalently with Clarithromycin on the surface of the *f*-SWCNTs. The amine loading was determined by applying the Kaiser test. The characterization of the developed nano-drug by transmission electron microscopy appeared good dispersibility of the functionalized single walled carbon nanotubes and separation of the nanotubes, which indicated the successful functionalization. Moreover, the percentage of functionalization was

determined by thermogravometric analysis obtaining 62% of functionalization in the case of *f*-SWCNTs **(16).** The antibacterial activity was determined by microdilution method through determining the MIC for four strains of bacteria, the result of the antibacterial activity indicated that the multi- functionalized SWCNTs have more significant increase of the antibacterial activity by 64 folds for three tested bacteria (*Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus,* and *Pseudomonas aeruginosa) and 16 folds for* (*Enterococcus faecalis)* in comparison with ciprofloxacin alone due to the presence of tetramine linker of *f*-SWCNTs **16**. Moreover, the *f*-SWCNTs (**15** and **16**) showed high hemocompatibility over a wide concentration range

Chapter One
Introduction

1.1 Infectious disease

Infectious diseases are disturbances caused via organisms as parasites, bacteria, fungi or viruses. Many of these organisms stay on and in our bodies. They're ordinarily helpful or harmless even, but under specific conditions, some organisms may source for disease [1]. Infectious diseases put to death more people worldwide than any other single cause. Infectious diseases are caused via germs. Germs are teeny living things that are found in water, air, and soil [2]. Before the discovery of antibiotics, people who sought the services of medical doctors for anything did, in fact, have a very high death rate from infection [3].

Penicillin was one of the world's premier antibiotics [4] was discovered by Alexander Fleming professor in London [4]. Penicillin is a class of drugs that struggle infections by keeping them from reproducing or killing bacteria. Since they were inserted in the 1940s, antibiotics have rescued millions of lives across the earth, and turn into one of the most heavily relied-upon therapies for infectious diseases [4].

There are now various sorts of antibiotics, the most of them can be categorized into six groups: Penicillins, Cephalosporins, Aminoglycosides, Tetracyclines, Macrolides and Fluoroquinolones. While each class is contained of numerous drugs, each drug is individual in some way [5].

The early quinolones as Piromidic acid, Cinoxacin, and Nalidixic acid were limited in the treatment of urinary tract infections caused by enteric bacteria and to remedy bacterial enteritis. The effective adjustment of the quinolone antibacterial agent, an entering of fluorine in the C-6 position, an expansion of an ethyl substituent at the N-1 position and a piperazine group at the C-7 position has produced the synthesis many of compounds. Ciprofloxacin was one of these compounds, that was proved to possess wide in vitro and in vivo antibacterial action against both Gram-negative and Gram-positive organisms [6].

1.2 Ciprofloxacin

Ciprofloxacin (CIP) is l-cyclopropyl-6-fluoro-l,4-dihydro-4-oxo-7-(1-piperazinyl)-quinoline-3-carboxylic acid [6]. Figure (1.1) demonstrates the chemical structure of CIP and table 1.1 summarizes the physicochemical properties of Ciprofloxacin.

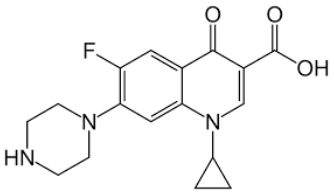


Figure.1.1: Ciprofloxacin chemical structure [6].

Table (1.1): The physicochemical properties of Ciprofloxacin [7].

Molecular formula	$C_{17}H_{18}FN_3O_3$
Molar mass	331.346 g/mol
Melting Point	255-257 °C
Water Solubility	30000 mg/L (at 20 °C)
$logP_{oct/wat}$	1.58
pKa values	6.09 and 8.74
Protein binding	20 to 40%
Half life	4 hours
Clearance	Renal Cl=300 mL/min

Ciprofloxacin is commercially obtainable as the monohydrate phase of CIP hydrochloride salt [8]. It is used for both intravenous and oral formulations and its minimum inhibitory concentration (MIC) susceptibility of 0.125–1µg/mL [9, 10]. Ciprofloxacin quickly blocks bacterial DNA replication during inhibiting DNA gyrase, that initial prokaryotic enzyme that one catalyzes chromosomal DNA supercoiling. In biological fluids, mainly CIP occurs as a zwitterion, which lets CIP to pass physiological barriers by passive diffusion, and CIP is highly active against a broad spectrum of microorganisms included aminoglycoside or lactam -resistant bacteria [11-13]. Less common effects may contain central nervous system events (less than 5%), renal disturbances (approximately 4.5%), blood disorders (approximately 5%), skin photosensitivity and hypersensitivity effects (approximately 2%). Rare occurrences of tendinitis, convulsions, and psychosis [14]. CIP has a bitter taste, it becomes a palatability difficulty and hasn't patients compliance during oral administration [9].

The extensive use of fluoroquinolones has led to rising resistance to these antimicrobials, with rates of resistance that differ by both geographic region and organism. Resistance to fluoroquinolones typically grows as a result of changes in the target enzymes (topoisomerase IV and DNA gyrase) and of alterations in drug efflux and entry [15].

Resistance to Fluoroquinolone has risen since their intro for urinary tract infection therapy. Various studies reported an evident increase in CIP resistance. Example, in Spain, it was 14.7% [16], and in Bangladesh, it was 26.0% [17]. High resistance to CIP was detected among *Escherichia coli* (12.0%), *Klebsiella pneumonia* (17.6%) *Pseudomonas aeruginosa* (20.0%), and *Staphylococcus saprophyticus* (25.0%) [17].

1.3 Clarithromycin

Clarithromycin is a macrolide antibacterial [18]. It is synthesized by substituting a methoxy group for the C-6 hydroxyl group of Erythromycin [19]. Figure 1.2 demonstrates the chemical structure of Clarithromycin and table 1.2 summarizes the physicochemical properties of Clarithromycin.

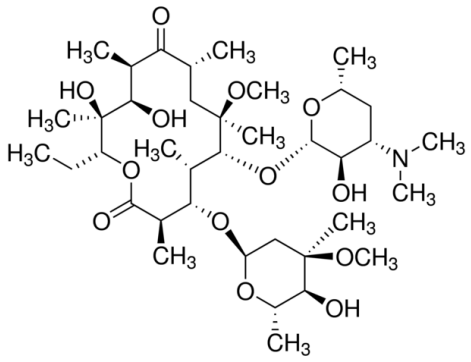


Figure1.2: Clarithromycin chemical structure [20]

Table (1.2): The physicochemical properties of Clarithromycin [21]

Molecular formula	$C_{38}H_{69}NO_{13}$
Molar mass	274.96 g/mol
Melting Point	222-225°C
Water Solubility	1.693mg/L (at 25 °C)
$logP_{oct/wat}$	3.16
pKa value	8.9
Protein binding	70%
Half life	3-4 hours
Clearance	Renal Cl= 6.7 to 12.8 L/h

Clarithromycin hinders bacterial protein synthesis by attaching to the bacterial 50S ribosomal subunit [22]. Clarithromycin may be bactericidal or bacteriostatic depending on the drug concentration and organism. This substitution makes a higher acid-stable antimicrobial and blocks the degradation of the Erythromycin base to the hemiketal intermediate. The raised acid stability of Clarithromycin results in reduced gastrointestinal intolerance and improved oral bioavailability. Clarithromycin is available as immediate-release tablets, extended-release tablets (250 or 500 mg) and granules for oral suspension (250 or 125 mg per 5 mL) [19]. Clarithromycin is applied to remedy infections of tonsils, skin, bronchioles, throat, lungs, larynx, and middle ear [23]. Clarithromycin mostly is well tolerated, and side effects usually are transient and mild. The side effects of Clarithromycin are a headache, abdominal pain, diarrhea, and nausea, abnormal taste, and dyspepsia. Other side effects, which are exceptional, but severe include seizures, hearing loss, abnormal heartbeats and liver failure [23].

Antibiotic-resistant bacteria are a dangerous threat to public health because of the slow expansion of new antibiotics to exchange those that become inefficient. Other new antimicrobial agents are required and a lot of research has been devoted to improving very effective compounds that are further less liable to the evolution of resistance by the bacteria. Cationic compounds have arisen as encouraging candidates for developments as antimicrobial agents with lowered potential for resistance development [24].

1.4 Polycationic polymers

Polycationic polymers are employed broadly for the transfer of material over cell membranes. Synthetic polymers ordinarily utilized involve branched polymers as poly(amidoamine) (PAMAM) dendrimers, and linear macromolecules as poly-L-lysine (PLL), polyethyleneimine (PEI). Interestingly, various classes of natural polycationic polymers seem to work an alike function, albeit by a various mechanism, inclusive the cell-penetrating peptides [25]. Cationic polymers poly(ethylene imine)s have been used as drug carriers in various application such as biomedical due to their high ability to enter cells or penetrate cell membranes, branched and linear PEIs (B-PEIs and L-PEIs) as shown figure 1.3. Moreover, most of researches have concentrated on the antibacterial activity of water-soluble PEI derivatives including (QAS) quaternized ammonium salt groups with long aromatic or alkyl groups and other applications for water-insoluble hydrophobic PEIs as antibacterial coatings, and nanoparticles [26].

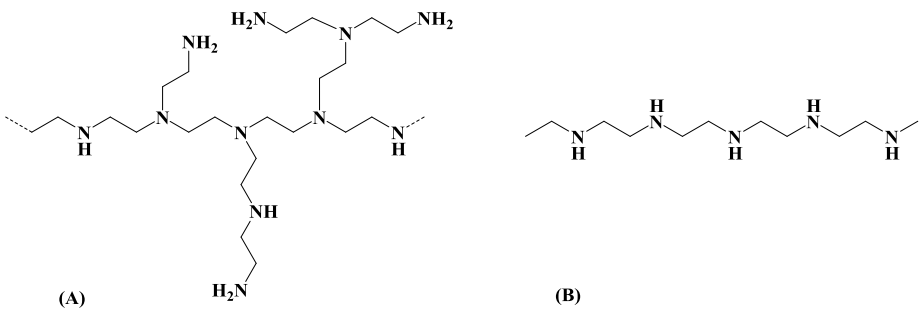


Figure (1.3): (A): Branched PEI (B-PEI) (B): Linear PEI (L-PEI) [26]**.**

Quaternary ammonium, have excellent processability and high charge density, presenting high antimicrobial activity [27]. Theoretically, negatively charged microorganisms completely attract the positively charged quaternary ammonium groups. Therefore, the higher electrostatic interaction will generate stronger adsorption to microorganisms, following in improved antibacterial activity and reduced resistance for these antibiotic [28]. Lin et al. determined that N-alkylated poly(ethyleneimine) may be active against a variety of Gram-positive bacteria and Gram-negative. Its antibacterial activity was observed to be dependent on molecular weight of the conjugate [29] and it seems that the Gram-negative bacteria were more sensitive to QPEI (quaternary polyethyleneimine) compared with the Gram-positive strains due to the substantial dissimilarity in the cell wall structure [30].

One promising way for selective treating of bacterial infections is to employ suitable nanomaterials. Nanomaterials have attracted tremendous attention over the past decennium because of their unique electronic, optical, mechanical, and chemical properties. Carbon nanotubes (CNT) are one of the interesting nanomaterials drug delivery system (DDS) that have

been developed for the strong biological carrier to deliver antibacterial and anticancer drugs [18]. In the following section we will discuss the properties of carbon nanotubes.

1.5 Carbon nanotubes

In reality, nanotubes had been discovered thirty years earlier. Nanotubes were observed as hollow, straight tubes of carbon that seem consist of graphitic layers of carbon detached by the same spacing as the planar layers of graphite. The present huge show interest in carbon nanotubes (CNTs) elemental carbon in the sp^2 hybridization is the direct result of the synthesis of fullerene carbon 60 (C60), and other types from fullerenes [31]. At 1990 that C60 could be generated in a simple arc evaporation device readily available in all labs. Japanese scientist "Sumio Iijima" discovered fullerene-regarding CNTs in 1991 [32]. CNTs are classified to multi-walled carbon nanotubes (MWCNTs) and single-walled carbon nanotubes (SWCNTs). As shown in figure 1.4.

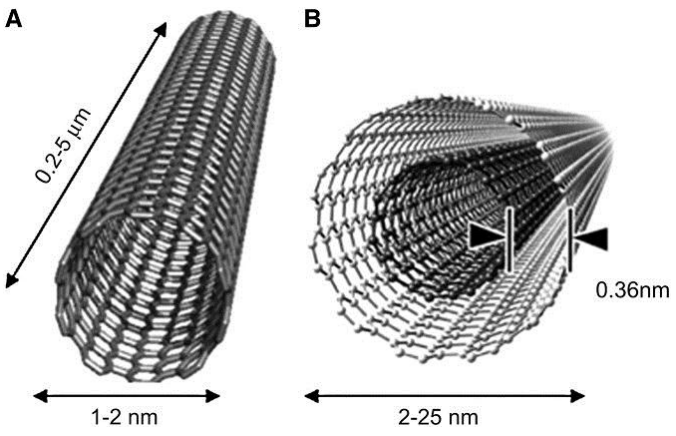


Figure (1.4): Classification of CNTs according to number of layers: A. SWCNTs B. MWCNTs [33].

A multi-walled carbon nanotubes is a bundle of graphene sheets wrapped into concentric cylinders, and extended in outer diameter from 3 to 30nm, and these closed at both ends [34]. On the other hand, in 1993, SWCNTs were discovered through the course of synthesizing nanocapsules carbon loaded with magnetic precise metal particles (Fe, Co, Ni). Nanotubes are contained in one wrapped graphene sheet rolled into the hollow cylinder [35, 36]. Table 1.3 demonstrates a comparison study between the SWCNTs and MWCNTs. In this study we used SWCNTs due to have large surface area in comparison with MWCNTs to get multi-functionalization with used combination therapy.

Table (1. 3): Comparison between SWCNTs and MWCNTs [36].

	SWCNTs	MWCNTs
1	Single layer of graphene	Multiple layer of graphene
2	Catalyst is required for synthesis	Can be produced without catalyst
3	Bulk synthesis is difficult as it requires atmospheric condition	Bulk synthesis is easy
4	Purity is poor	Purity is high
5	A chance of defect is more during Functionalization	A chance of defect is less but once occurred it‘s difficult to improve
6	Less accumulation in body	More accumulation in body
7	It can be easily twisted and are more pliable	It cannot be easily twisted

1.6 Functionalization of carbon nanotubes.

CNTs in all their sorts are difficult to dissolve and disperse in organic media and in water [37]. According to Hirsch, the various strategies to the functionalization of SWCNTs may do to categorize into five classes. The most common types are covalent and noncovalent functionalization as shown in Figure 1.5.

The overall aim of functionalizing Carbon Nanotubes for biomedical application is to increase their dispersion or solubility in biocompatible (aqueous) media, herewith reducing toxic effects [38].

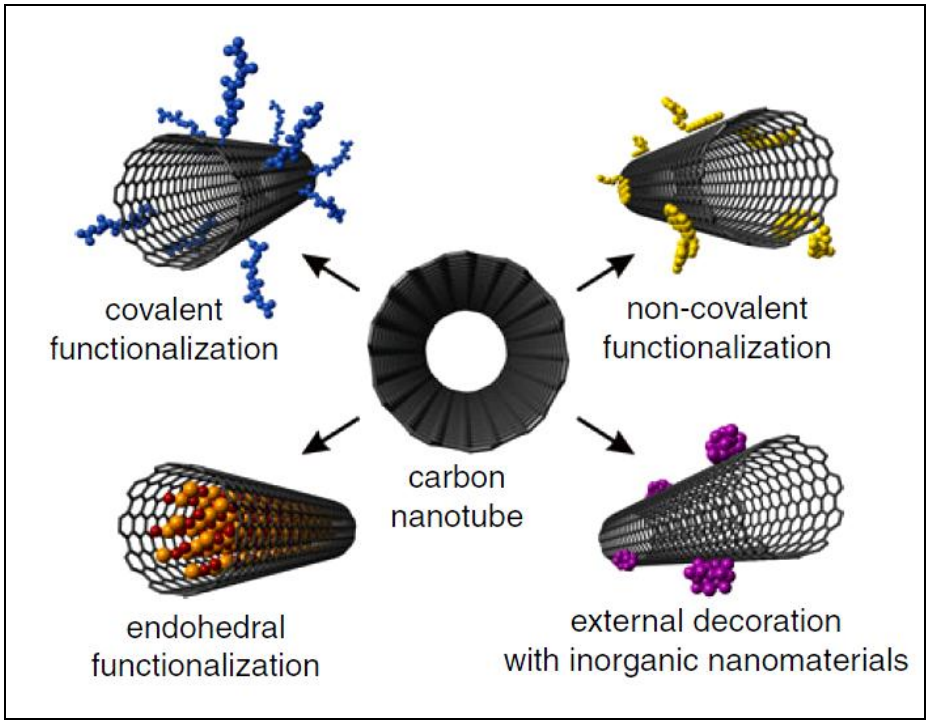


Figure (1.5): Schematic representation of different strategies for the functionalization of carbon nanotubes. Reproduced from ref.[39].

1.6.1 Non-Covalent functionalization.

In general, this type of functionalization occurs when CNTs interact or wrapping with a polymer, surfactant or aromatic compounds via weak Van der Waals (VdW) interactions, electrostatic interactions, or pi-pi stacking, as shown in figure 1.6. Non-covalent functionalization with conjugated organic molecules or polymers is attractive because chemical groups are linked without damaging or changing nanotubes structure from sp^2 to sp^3 and thus their electronic characteristics. After that, CNTs can be dispersed and enhanced solubility in organic or aqueous solution [40-42]. But it remains sensitive to environmental conditions such as pH and salt concentration. Accordingly, the release of the drugs that were charged on the surface of CNTs may occur before reaching the target site [40].

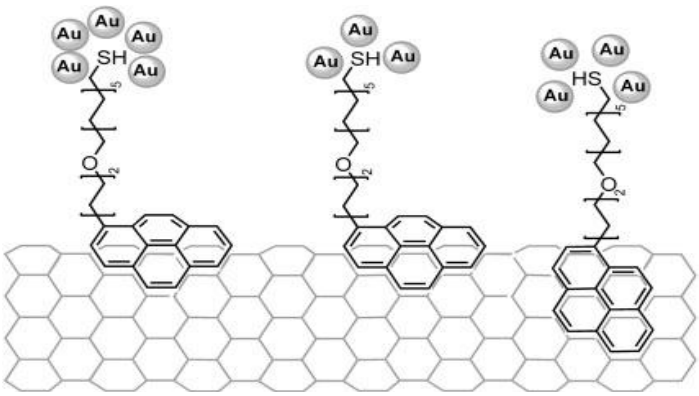


Figure (1.6): pi-pi stacking of 17-(1-pyrenyl)-13-oxaheptadecanethiol(PHT) with SWCNTs and self-assembling of gold nanoparticles from a colloidal gold solution [43].

1.6.2 Covalent functionalization.

Covalent functionalization is composed of the chemical covalent bond between functional groups and the surface of carbon nanotubes. It can be formed at the end caps or at sidewalls of nanotubes. When applied covalent sidewall functionalization, it is changing structure nanotubes from sp^2 to sp^3. This functionalization can be applied by reaction with some of functional groups or molecules have high chemical reactivity [37] such as fluorination [44], radicals addition, cycloaddition [45], carbenes, oxidation reaction [46], and others, as shown in figure 1.7.

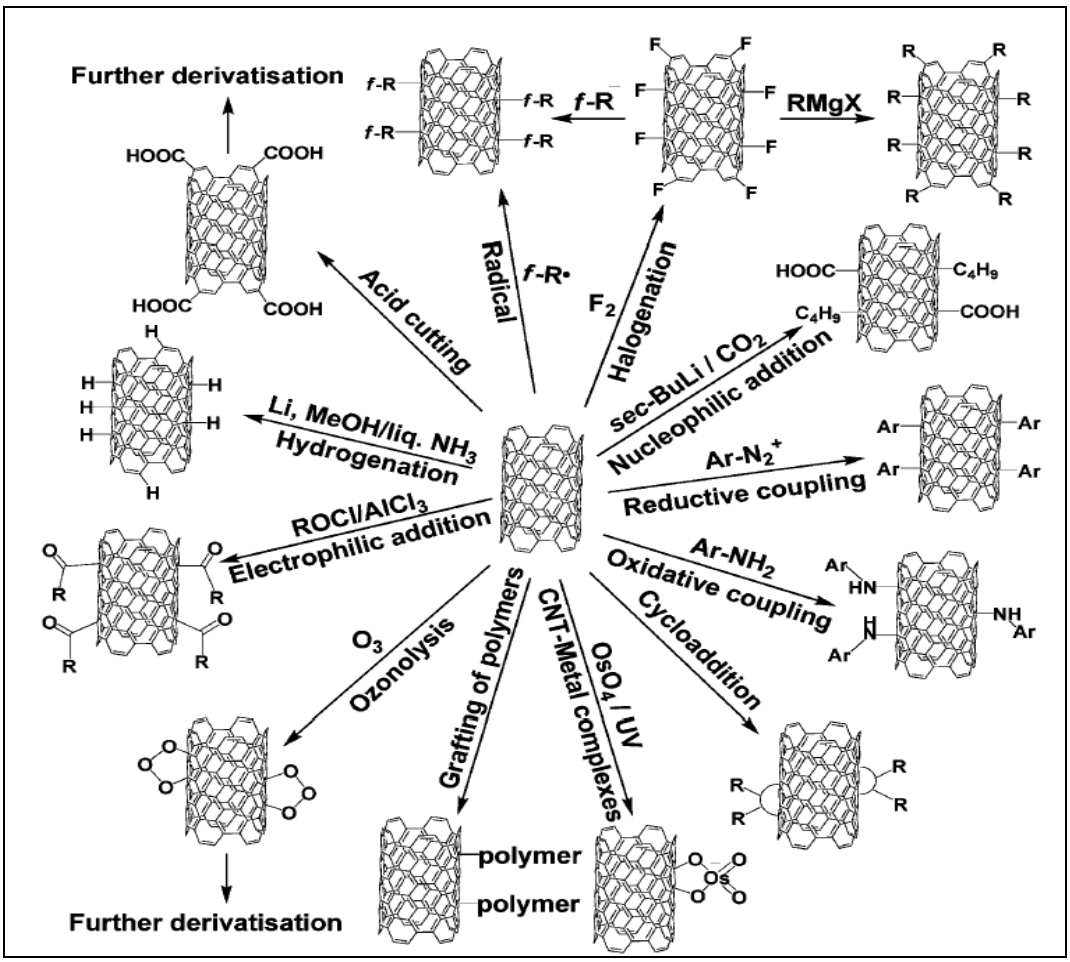


Figure (1.7): Covalent functionalization of carbon nanotubes [47].

Furthermore, covalent functionalization has been improved to produce water-solubility nanotubes, and helpful in certain biomedical usages such as drug delivery [48, 49]. In contrast, that covalent functionalization of CNTs is that the original properties and structure of CNTs are changed after alteration. Mostly, the more alteration on the surface, the more the outstanding properties of CNTs will be modified [50]. In the following section, the oxidation functionalization and the addition reaction will be discussed.

1.6.2.1 Oxidation reaction.

The oxidation of CNTs happens in strong oxidative and acidic conditions as combinations of strong acids incorporated with sonication or heating that allowed shortened open tubes decorated with oxygenated functions, predominant on the tips. Among the groups introduced on it, for example (carbonyl, hydroxyl, carboxyl, etc.), the functions are especially used as anchor points for esterification and amidation reactions as shown figure 1.8 [51].

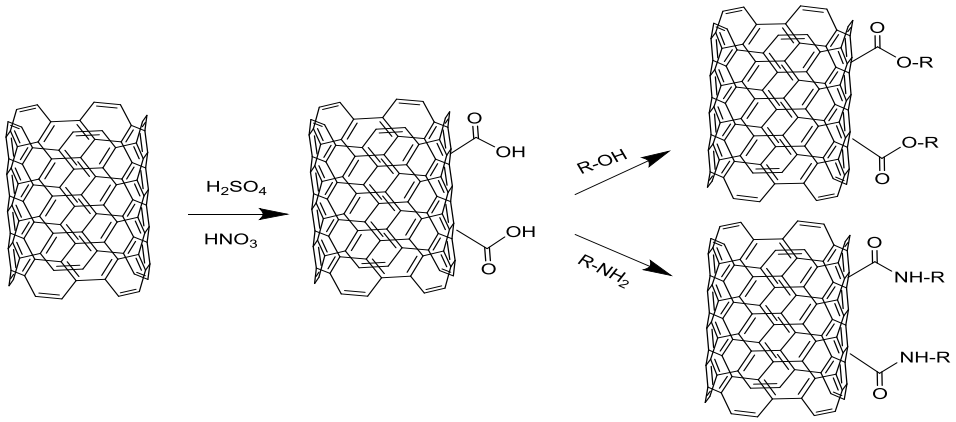


Figure (1.8): Oxidation CNTs, esterification and amidation of oxidized CNTs [52].

1.6.2.2 Addition reaction.

This reaction causes functionalization of carbon nanotubes by changing the hybridization of carbon atom from sp^2 to sp^3 of the predominantly trigonal-planer local bonding geometry into a tetrahedral geometry. There are different types of addition reactions such as fluorination, cycloaddition, radical additions, nucleophilic and electrophilic additions [51]. Tour and co-workers functionalized SWCNTs with reduced aryl diazonium salts via electrochemical reaction. The aryl radicals were generated from the diazonium salts by one-electron reduction [53]. The obtained materials showed good-dispersibility in both organic and water solvents as shown figure 1.9 [54].

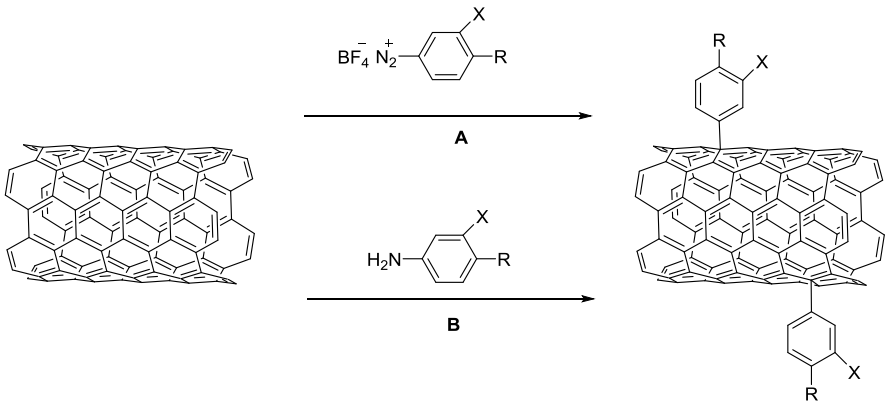


Figure (1.9): Functionalization of CNTs using diazonium coupling reactions under different conditions. A) In presence of surfactants or ionic liquids or electrochemical reaction. B) diazonium salt generated in situ with nitrites [52].

1.7 Literature review

Many attempts were carried out in order to improve the efficacy and safety of the antibacterial agents using different types of nano-systems such as nanoparticles, liposomes, micelles, and others.

Regarding to polymeric nanoparticles as nanoantibiotics to deliver ciprofloxacin, several studies were carried out. Among these studies ciprofloxacin was encapsulated in polyisobutylcyanoacrylate (PIBCA) nanoparticles by Fawaz *et al.* They studied their pharmacokinetic parameters after I.V. infusion using rabbits as animal model. The results revealed that ciprofloxacin –loaded PIBCA/NP lead to increase the *AUC*, $T_{1/2}$ and V_d, and to a decrease the *Cl* as compared with free form of ciprofloxacin. In addition, they tested these nanoparticles against a M. avium infection in a human macrophage culture. It was found that ciprofloxacin-loaded PIBCA/NP was more effective than unbound ciprofloxacin. In spite of this, the efficacy of ciprofloxacin associated with nanoparticles was much lower than anticipated due to the cytotoxicity of the polymeric material that was observed at concentrations higher than 80 mg of PIBCA per ml [55].

In 2010, Banoee studied the effect of zinc oxide (ZnO) nanoparticles prepared by a mechanochemical method on the antibacterial activity of various antibiotics (Ciprofloxacin, Amoxicillin, Penicillin G, and Nitrofurantoin).. Their results revealed that antibacterial activity of Nitrofurantoin, Amoxicillin and Penicillin G against *S. aureus* decreased,

while the antibacterial activity of Ciprofloxacin was increased in the presence of ZnO nanoparticles in both test strains [37].

In 2011, Zawrah, M. and S. Abd El-Moez have synthesized conjugated CIP gold nanoparticles. They have studied the antibacterial activity of these nanoparticles that were conjugated to CIP against *S. Typhimurium*, *B. cereus*, *E.coli*, *P. aeruginosa* and *L. monocytogenes*, and *S. aureus* by using disk diffusion and broth microdilution methods. It was clarified by the decrease in MIC of CIP when was conjugated to gold nanoparticles compared to free CIP [37].

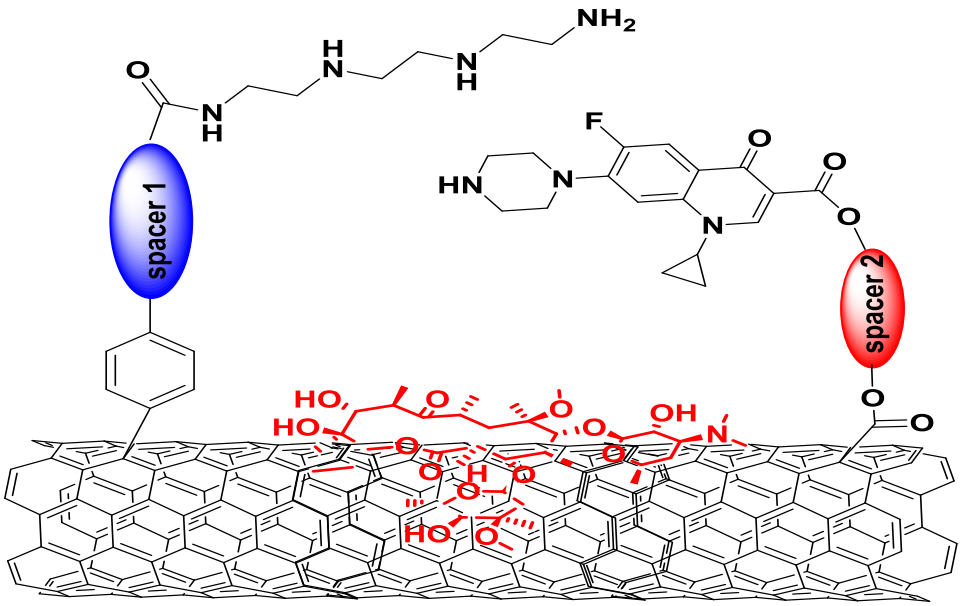
In another study, Liu et al. have prepared CIP liposomes submitting sustained in vitro release in simulated lung fluid, liposomes were administered to rats by intratracheal instillation. The drug concentration in the lung was higher for the liposomal antibiotic than for the free drug. Liposomal CIP presenting 7.21-fold in half-life increases over those of the free drug. Moreover, bioavailability results as well prove that liposomal CIP was capable to reach the lung and supply high drug concentrations at the target site. Moreover, an in vivo pulmonary irritation test offered CIP liposomes were capable to decrease alteration and irritation of the lungs after intratracheal instillation in rats [56].

In 2015, Farah Abdulla has synthesized CIP with covalent functionalized single walled carbon nanotubes (f-SWCNTs) by using radical reaction and CIP esterified using hexaethylene glycol linker, which was used to improve the water dispersibility. All the results of the characterization method were

performed the successful covalent functionalization of the SWCNTs. The effect of *f*-SWCNTs was tested against some resistant bacteria strains such as *Escherichia coli*, *Staphylococcus aureus*, and *P.aeruginosa*. Their results were an improvement in antibacterial activity (MIC) of the *f*-SWCNTs against the three tested strains of bacteria in comparison with CIP and *P*-SWCNTs alone. The improvement was 16 folds against *S.aureus* and *P.aeruginosa* and 8 folds for *E.Coli.* Moreover, the synthesized nanoantibiotic showed high hemocompatibility and cytocompatibility over a wide concentration range [7].

1.8 Aims of the study

The aim of our study is to develop a new nano-antibacterial system based on combination therapy using covalent functionalization of SWCNTs-COOH with CIP and the adsorption of Clarithromycin on the surface of the SWCNTs. Moreover, multiamine (cationic chain) was introduced in order to improve the penetration of the nanosystem and increase antibacterial activity as shown in the scheme 1.



Scheme (1): The general approach of the thesis aim

1.9 Objectives

1) Multi-functionalization of SWCNTs covalently with multiamines and Ciprofloxacin and non-covalently with Clarithromycin.

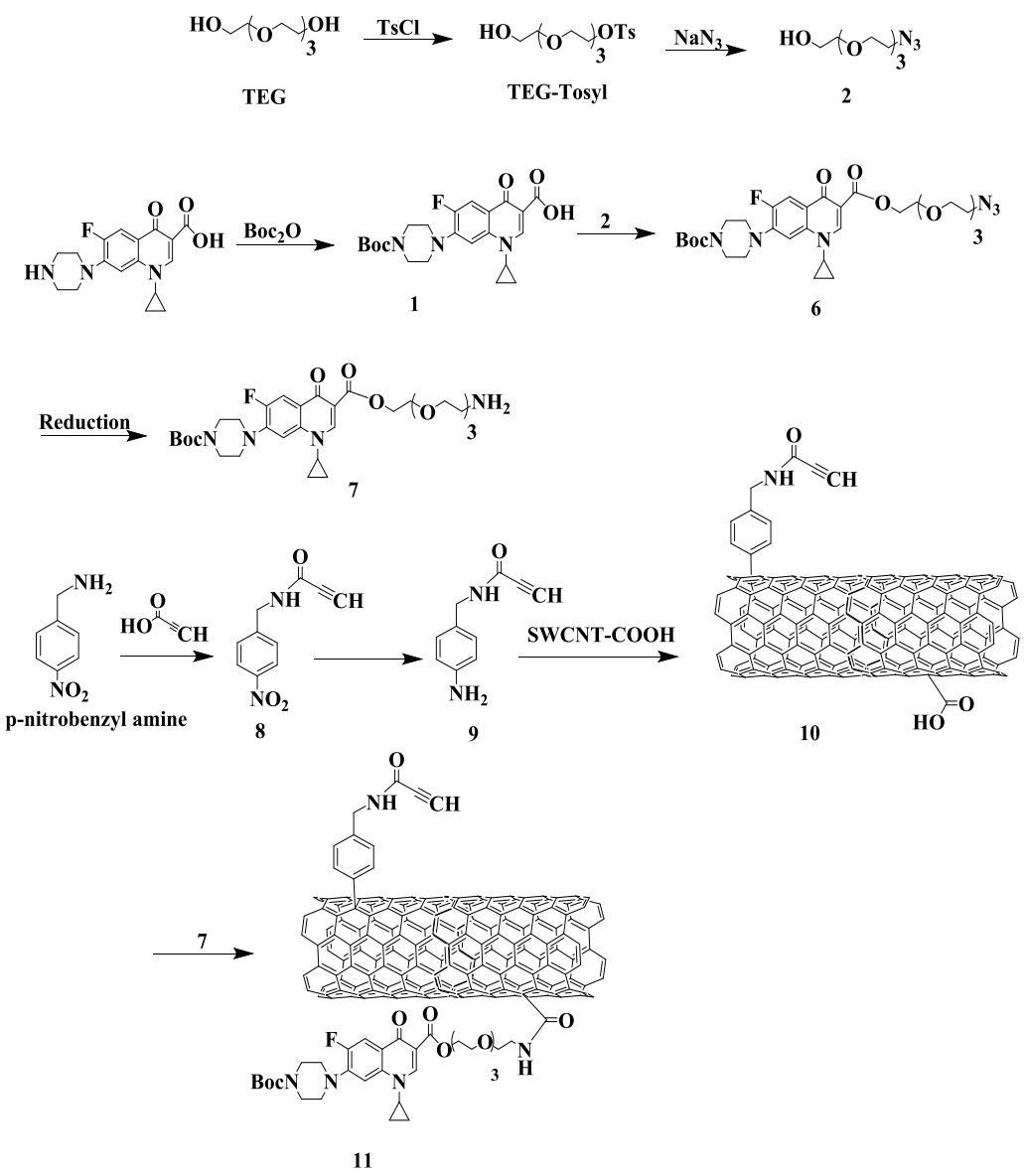
2) Characterization of the formed nano-antibiotic by the different analytical techniques such as NMR, UV-Vis spectroscopes, TEM and TGA.

3) The determination of the antibacterial activity by *in vitro* test and the comparison with the Ciprofloxacin and Clarithromycin alone.

4) Determine the hemocompatibility of the synthesized nanoantibiotic.

1.10 General approach of the synthesis and functionalization of SWCNTs

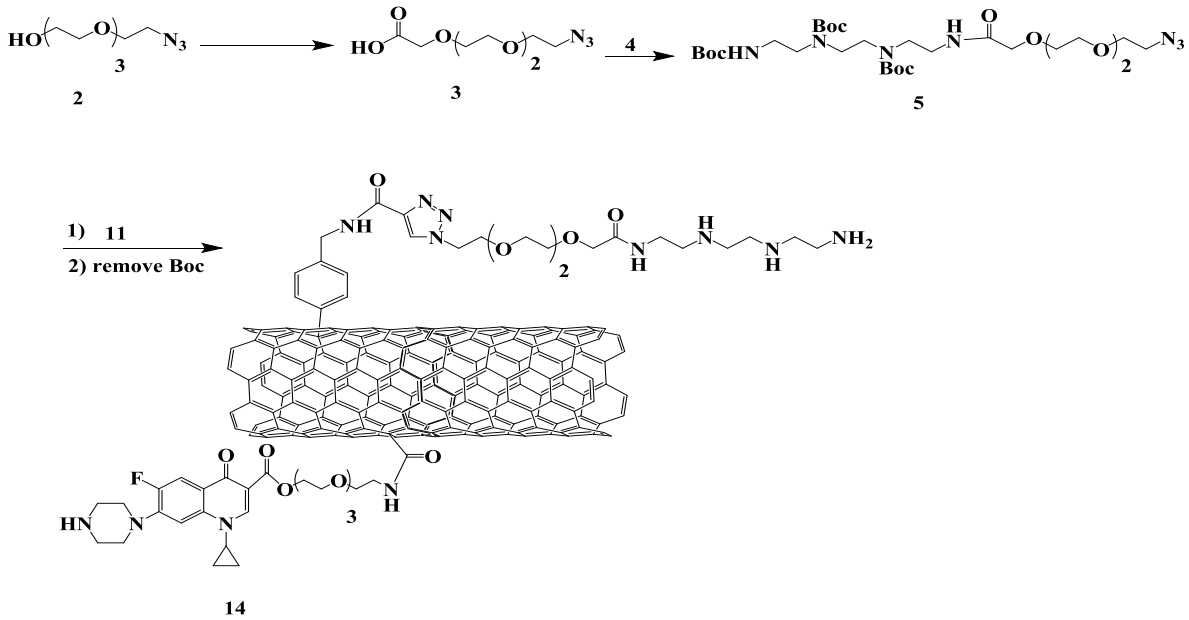
In order to obtain the multifunctionalization of SWCNTs, multi steps were conducted beginning in the attachment of the CIP followed by the tetramine linker and finished by the noncovalent adsorption of Clarithromycin. Scheme 2 summarizes the attachment of CIP to the functionalized SWCNTs-COOH through hydrolysable linkage. This was obtained through multistage process that started with the esterification reaction of OH-TEG-N_3 with *N*-Boc Ciprofloxacin **(1)** to get compound **(7).** The linker was synthesized by tosylation of tetraethyelene glycol (TEG), and replaced with NaN_3. In parallel; functionalized SWCNTs **(10)** was obtained by the reaction between 4-aminobenzylamine **(9)** and carboxylated-SWCNTs through Tour reaction. The obtained alkyne-SWCNTs **(10)** were linked with compound **(7)** through amidation reaction to get *N*-Boc Ciprofloxacin-alkyne-SWCNTs **(11)**.



Scheme (2): The functionalization of alkyne-SWCNTs with *N*-Boc Ciprofloxacin

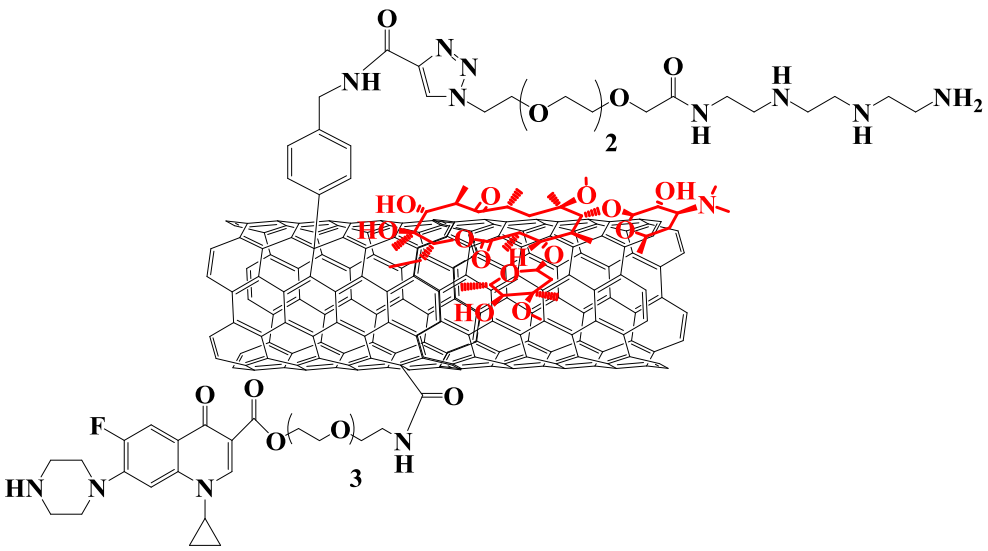
Scheme 3 showed the attachment of tetramine linker to the *f*-SWCNTs. The OH-TEG-N_3 was oxidized to COOH-TEG-N_3 in order to react with teteramine-Boc. An amidation reaction was used to attach linker **(3)** with Boc protected tetramine. The obtained alkyne-SWCNTs **(11)** were linked with compound (**5)** through Click reaction to get the dual functionalized SWCNTs. After that**,** compound **(13)** was deprotected from Boc group to

get *f*-SWCNTs **(14)**. Following, a multi-functionalization SWCNT, this was obtained through *f*-SWCNTs **(14).**



Scheme (3): The dual functionalization of SWCNTs with Ciprofloxacin and cationic tetramine linker.

In final stage, Clarithomycin was adsorbed on the surface of the *f*-SWCNTs (**14)** noncovalently to get *f*-SWCNTs (**16).** As shown in scheme 4.



Scheme (4): The multi functionalization of SWCNTs with Ciprofloxacin, cationic tetramine-linker and Clarithromycin

Chapter Two
Reagents and Methods

2.1 Materials

Ciprofloxacin powder 98% (catalog # J61317), toluene-4-sulfonyl chloride (catalog # 89732), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (catalog # A10807), tetraethylene glycol (catalog # B23990), L-ascorbic acid sodium salt (catalog # A17759) and trifluoroacetic acid (TFA) (catalog # A12198) were purchased from (Alfa Aesar Company, England). 1, 4-dioxane (catalog # 33147), 1, 2-Dichlorobenzene (*o*-DCB) (catalogue # 65152), solvents were purchased from (Riedel-de Haën Company, Germany). Isoamyl nitrite (catalog # 110463), sodium azide (catalog # 0E30428), N,N-diisopropylethylamine (DIPEA) (catalog # 496219), ethyl trifluroacetate (catalog # A821163), 4-(dimethylamino) pyridine (DMAP) (catalog # 1122583), 4-nitrobenzylamine hydrochloride (catalog # 191434), palladium on carbon (catalog # 101375286), anhydrous copper sulfate (catalog # 451657), di-tert-butyl dicarbonate (BOC_2O) (catalog # 101281549), and Silica gel were purchased from (Sigma-Aldrich, USA). SWCNT-COOH was brought from (carbon solutions, USA). Tween 20 (catalog # E0088) was purchased from sun-pharm drug store (Nablus). Disodium hydrogen phosphate, potassium dihydrogen phosphate, Methanol (MeOH), dichloromethane (DCM), Isopropyl alcohol, Acetone and ethanol were purchased from (C.S. Company, Haifa). Ethyl acetate (EtOAc) (catalogue # 2355516100024), n-hexane (Hex) (catalogue # 2355544800024) and acetonitrile (CH_3CN)

(catalogue # 5550070) solvents were purchased from (Frutarom Company, Haifa). Chloroform ($CHCl_3$) (catalogue # 67-66-3) was purchased from (merckmillipore) and tetrahydrofuran (THF) solvent (catalogue # 487308) was purchased from (Carlo Erba Company, MI. Italy).

TLC (DC-FertigfolienAlugeram®Silg/Uv_{254}, Macherey Nagel Company, Germany) was used to monitor the reactions, aqueous phosphomolybdic acid hydrate (catalog # 221856), BBL™ Muller Hinton Broth (catalog # 212322) were purchased from (BD Company, USA).

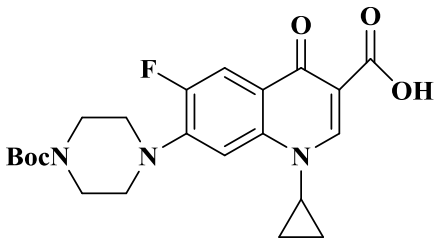
2.2 Equipments

UV/Vis absorption analysis was measured on (Jenway, 7315 Spectrophotometer, UK) using 10-mm quartz cuvettes. Accumax Variable micropipette, UK was used for pipetting. NMR analysis was recorded by Bruker Avance 500 spectrometer at Jordan University. TEM images were achieved on FEI Morgagni at Jordan University. FTIR analysis was taken on Nicolet iS5, ThermoFisher Scientific Company, USA. TGA analysis was measured on STA instrument at Jordan University with a flow rate 20 ºC under nitrogen (100 cc/min) with a range 20-600 ºC. Water bath and Sonicator (Elmasonic S 70 H, Elma®, Germany). Rotary Evaporator (MRC, ROVA-100, laboratory equipment manufacturer). Centrifuge (UNIVERSAL 320, HettichZentrifugen, Germany). Stat Fax® 2100-Microplate reader, Awareness Technology INC, FL, USA was used at λ max 630 nm for measuring bacterial viability.

2.3 Synthesis and characterization of the products:

All the synthetic steps and antibacterial activity were conducted at An-Najah University laboratories. NMR measurements, TEM and TGA measurements were conducted at the University of Jordan.

2.3.1 Synthesis of (7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) (1)

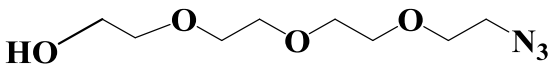


Ciprofloxacin (2.0 g, 6.0 mmol), 60 ml mixture of (water: dioxane) (1:1) and 9 ml 1M NaOH were added to RBF. Then Boc_2O (2.0 g, 9.2 mmol) was added to this reaction, and the RBF allowed working on stirrer to completion. Then the solvent was evaporated under vacuum by rotary evaporator. After that, 40 ml of acetone was added. The product was filtered using vacuum filtration, washed by 30 ml acetone and dried under high vacuum. Column chromatography with a mobile phase (DCM: MeOH (20:1)) was used to obtain pure product. Then product was obtained as white to pale- yellow solid. Yield (85%) (2.2 g, 5.0 mmol).

$\mathbf{R}_f$: 0.4 (DCM: MeOH (9:1)).

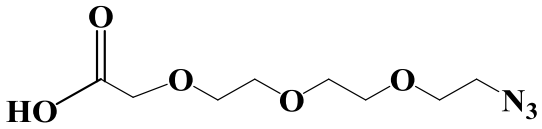
The characterization confirmed the obtaining of the product as published previously [7].

2.3.2 Synthesis of (2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol) (2)



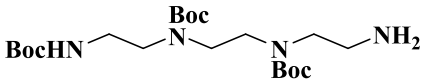
Compouhd **(2)** was synthesized as published previously in our research group [52].

2.3.3 Synthesis of (2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetic acid) (3)



Compouhd **(3)** was synthesized as published previously in our research group [52].

2.3.4 Synthesis of (tert-butyl(2-((2-aminoethyl) (tert butoxycarbonyl) amino) ethyl) (2((tertbutoxycarbonyl)amino)ethyl) carbamate) (4)



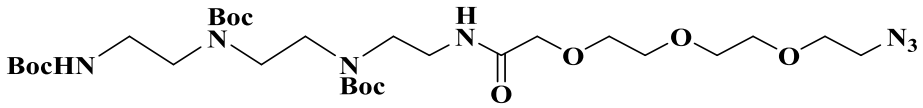
A volume of 70 ml dry methanol was added to triethylenetetramine (745 µl, 4.99 mmol) under argon. Ethyl trifloroacetate (995 µl, 4.99 mmol) was added for reaction drop by drop for 45 min on dry ice (-78 ºC). After that, remained in dry ice for another 45 min. Then the reaction was remained for an hour at 0 ºC. Boc_2O (4.0 g, 18.3 mmol) in 10 ml dry methanol was added dropwise, the re0action remained on stirrer overnight. Methanol was evaporated and the powder was diluted with 60 ml DCM. Then the reaction was extracted by a solution of 100 mg $NaHCO_3$ in 40 ml distilled water and Dichloromethane layer was dried using (Na_2SO_4), filtered and evaporated.

The product was white powder. TLC was visualized by (DCM: MeOH: NH_4OH (10:1:0.1)), yield (72%), (1.8 g, 8.2 mmol). After that, product was crystallized using DCM/Hexane to obtain white solid. Then the powder was dissolved in 70 ml methanol and (580 mg K_2CO_3 in 5 ml distilled water). The reaction was running on stirrer at 70 ºC for 6 hours. Methanol was evaporated and the reaction was extracted with 150 ml of dichloromethane and 50 ml of distilled water. Dichloromethane dried by using (Na_2SO_4), then filtered and evaporated. The reaction was concentrated and purified by using silica gel column chromatography (DCM: MeOH: NH_4OH (10:1:0.1)) to provide triethylenetetramine-Boc compound. Yield (44 %) (1.0 g, 2.24 mmol).

R_f: 0.48 (DCM: MeOH: NH_4OH (10:1:0.1)).

1H NMR (500 MHz, $CDCl_3$): δ 3.35 – 3.20 (bm, 10H, $4CH_2N$ & CH_2NH), 2.68 (bt, 2H, CH_2NH_2), 1.41 (s, 27H, $C(CH_3)_3$), 1.35 (s, 2H, NH_2).

2.3.5 Synthesis of (tert-butyl (17-azido-3-(tert-butoxycarbonyl)-7-oxo-9,12,15trioxa3,6diazaheptadecyl)(2((tertbutoxycarbonyl)amino)ethyl)carbamate) (5)



To a solution of compound **(4)** (180 mg, 0.4 mmol) and DIPEA (86 mg, 0.67 mmol) under argon, a solution of compound **(3)** (100 mg, 0.4 mmol), DIPEA (86 mg, 0.67 mmol) and TBTU (169 mg, 0.44 mmol) was added in acetonitrile. Then reaction was stirred at room temperature for 24 hr. The

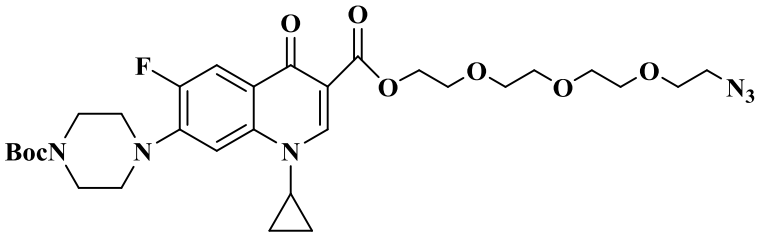
reaction was extracted with 70 ml of dichloromethane and 30 ml of 1M HCl. Organic layer was dried over (Na_2SO_4). The product was pale yellow oil. TLC was visualized by (DCM: MeOH (15:1)). (240 mg, 0.36 mmol) yield (89%)

R_f: 0.5 (DCM: MeOH (15:1)).

IR: 2105.28, 3345.60, 2928.01, 1688.25 cm^{-1}.

1H NMR (500 MHz, $CDCl_3$): δ 3.96 (s, 2H, CH_2CONH), 3.68-3.64 (m, 8H, $4CH_2O$), 3.39-3.24 (m, 16H, $4CH_2N$, $2CH_2NH$, $COCH_2CH_2N_3$ & $COCH_2CH_2O$), 2.2 (t, 2H, J = 4.4 Hz, CH_2N_3), 1.44 (s, 27H, $C(CH_3)_3$).

2.3.6 Synthesis of (2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl 7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate) (6)



To compound **(1)** (300 mg, 0.7 mmol), compound **(2)** (183 mg, 0.834 mmol), EDC (267 mg, 1.39 mmol), and DMAP (85.3 mg, 0.7 mmol) were added under argon in 10 ml dry dichloromethane. The reaction was stirred at room temperature for 96 hour. The reaction was extracted by 40 ml brine and 60 ml DCM. The organic phase was collected and dried using Na_2SO_4, then evaporated. Then column chromatography with a mobile phase of

DCM: MeOH (15:1) was conducted to obtain pure color yellow oil with a yield (50%), (220 mg, 0.34 mmol).

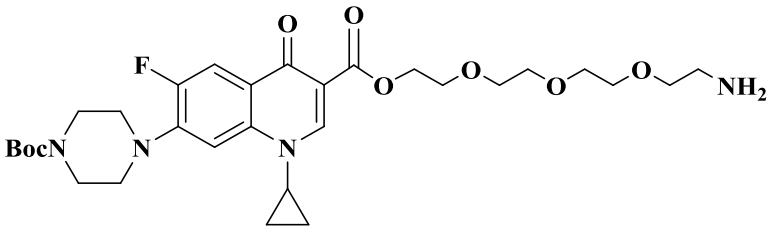
R_f: 0.6 (DCM: MeOH (15:1)).

IR: 1727.69, 2103.95, 2854.07 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.54 (s, 1H, C-C<u>H</u>-N of pyridine ring), 8.06 (d, 1H, *J* =12.8 Hz, 1Ar), 7.33-7.26 (m, 1H, 1Ar), 5.34-5.28 (m, 1H, CH of cyclopropyl), 4.39 (t, 2H, *J* = 4.6 Hz, CH_2OCO), 3.77 (t, 2H, *J* = 4.9 Hz, C<u>H$_2$</u>CH_2OCO), 3.61-3.55 (m, 10 H, $5CH_2O$), 3.34-3.15 (m, 10H, $4CH_2$ of piperazine ring, CH_2N_3), 1.43 (s, 9H, $3CH_3$), 1.25-1.07 (m, 4H, $2CH_2$ of cyclopropyl).

^{13}C NMR (125.7 MHz, $CDCl_3$): δ 165.3, 154.61, 148.2, 138.0, 129.0, 123.3, 113.6, 110.1, 105.1, 80.4, 80.2, 72.5, 70.7, 70.6, 70.4, 70.1, 70.0, 69.2, 63.8, 61.8, 50.7, 34.5, 31.9, 29.7, 28.4, 22.7, 14.1, 8.2.

2.3.7 Synthesis of 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl 7-(4-(tert-butoxycarbonyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (7)



A weight of 12 mg of Pd/C (10%) was added to solution of compound **6** (120 mg, 0.19 mmol) in 8 ml acidic methanol (methanol with 5 drops of 1 M HCl) under hydrogen gas (1 bar). Then, the mixture was stirred for 24 hr at room temperature under hydrogen gas. After that, methanol was evaporated. The reaction was filtrated and washed with dichloromethane. Then DCM evaporated. The product was pale yellow oil. Yield (87%) (100 mg, 0.165 mmol).

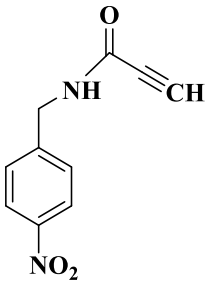
R_f: 0.3 (DCM: MeOH (15:1)).

IR: 3374.09, 2924.94, 1700.48 cm^{-1}.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.56 (s, 1H, C-C<u>H</u>-N of pyridine ring), 8.12 (d, 1H, *J* =12.8 Hz, 1Ar), 7.29-7.23 (m, 1H, 1Ar), 4.94-4.62 (m, 1H, CH of cyclopropyl), 4.29 (t, 2H, *J* = 4.6 Hz, CH_2OCO), 3.87 (t, 2H, *J* = 4.9 Hz, C<u>H$_2$</u>CH_2OCO), 3.66-3.59 (m, 10 H, $5CH_2O$), 3.34-3.15 (m, 10H, $4CH_2$ of piperazine ring, CH_2N_3), 1.95 (s, 2H, NH_2), 1.45 (s, 9H, $3CH_3$), 1.23-1.08 (m, 4H, $2CH_2$ of cyclopropyl).

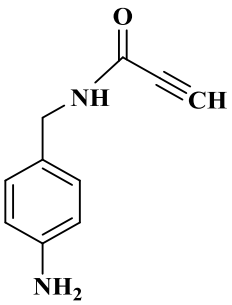
^{13}C NMR (125.7 MHz, $CDCl_3$): δ 166.3, 154.71, 152.1, 148.2, 144.2, 129.0, 123.3, 112.6, 111.1, 106.1, 80.5, 80.3, 72.5, 70.7, 70.6, 70.4, 70.1, 70.0, 69.2, 63.8, 61.8, 52.7, 40.5, 31.9, 29.7, 28.4, 14.1, 8.2.

2.3.8 Synthesis of (N-(4-nitrobenzyl) propiolamide) (8)



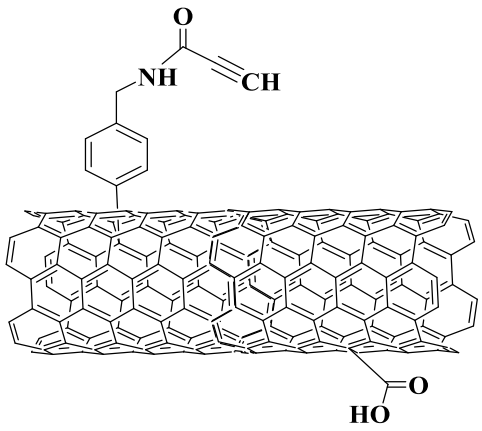
The synthesis was achieved according to a previous thesis work in our research group [57].

2.3.9 Synthesis of (N-(4-aminobenzyl) propiolamide) (9)



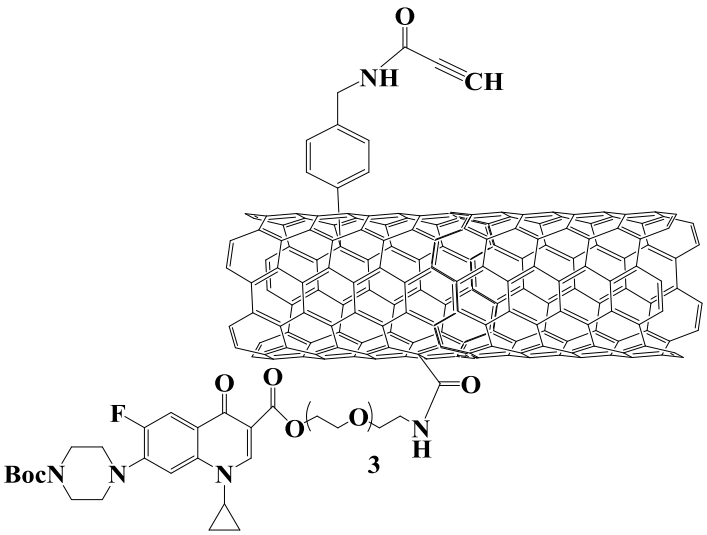
The synthesis was achieved according to a previous thesis work in our research group [57].

2.3.10 Functionalization of carboxylated SWCNTs with compound 9 (10)



Carboxylated SWCNTs (50 mg), compound **(9)** (100 mg, 0.574 mmol) were solubilized in *o*-DCB (13 ml), CH_3CN (7 ml) under argon. The reaction was sonicated for 15 min, and then the isoamylnitrite (463.3 µl, 0.82 mmol) was added drop by drop. The solution was stirred for 24 hr at 60ºC. After cooling the reaction to room temperature, the reaction was sonicated for 15 min. Unfunctionalized SWCNTs were removed by high vacuum filtration, then that, functionalized SWCNTs were washed with 30 ml MeOH, 30 ml dichloromethane, and diethyl ether (2 x 30 ml). The functionalized SWCNTs were dried under vacuum. Then resulting black product was collected and weighed, the yield compound **(10)** (72 mg).

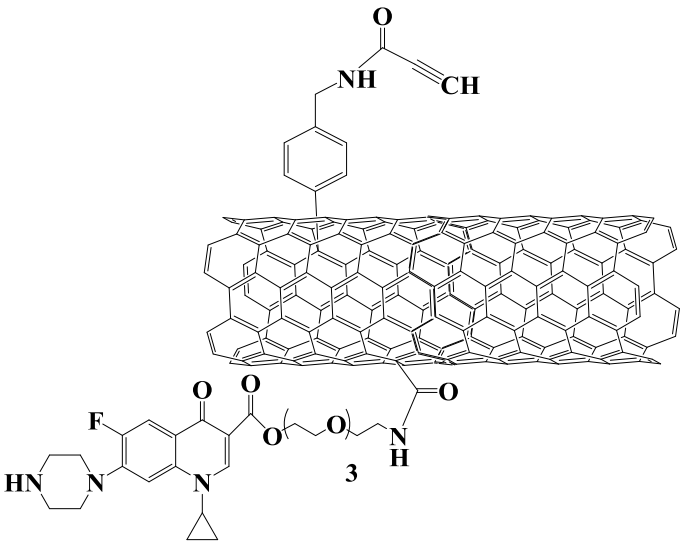
2.3.11 Functionalization of SWCNTs (10) with compound 7 (11)



A volume of 5 ml of dry DCM was added to *f*-SWCNTs (**10)** (50 mg), EDC (21.5 mg, 0.112 mmol) and DMAP (3.4 mg, 0.028 mmol) under argon. The reaction mixture was sonicated for 5 min, and then stirred for 30 min. Then a solution of compound **(7)** (200 mg, 0.33 mmol) in DCM (4 ml) and DMAP (3.4 mg, 0.028 mmol) under argon was added to *f*-SWCNTs

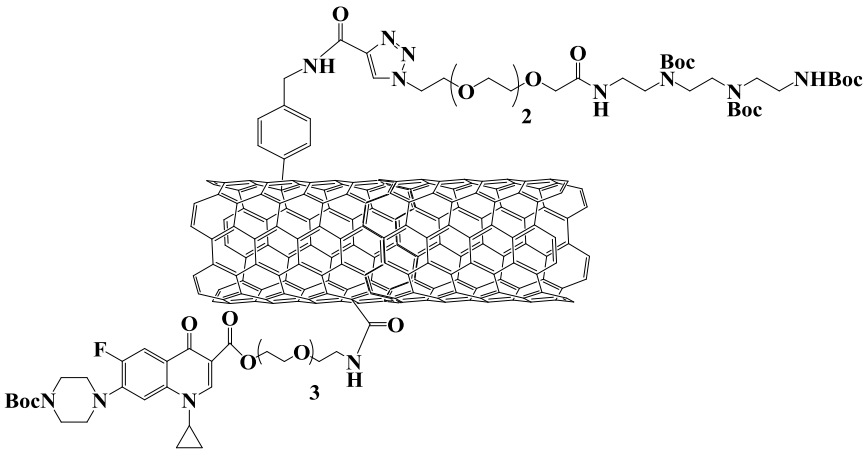
(**10)** suspension. The reaction was allowed to stirrer for 24 hr under argon. 15 ml of MeOH was added to reaction and sonicated for 10 min, then filtered by vacuum. Washing steps were refined with MeOH (20 ml), and ether (40 ml). After that, Black powder was dried obtaining 48 mg of the product.

2.3.12 Deprotection of BOC group in *f*-SWCNTs 11 (12)



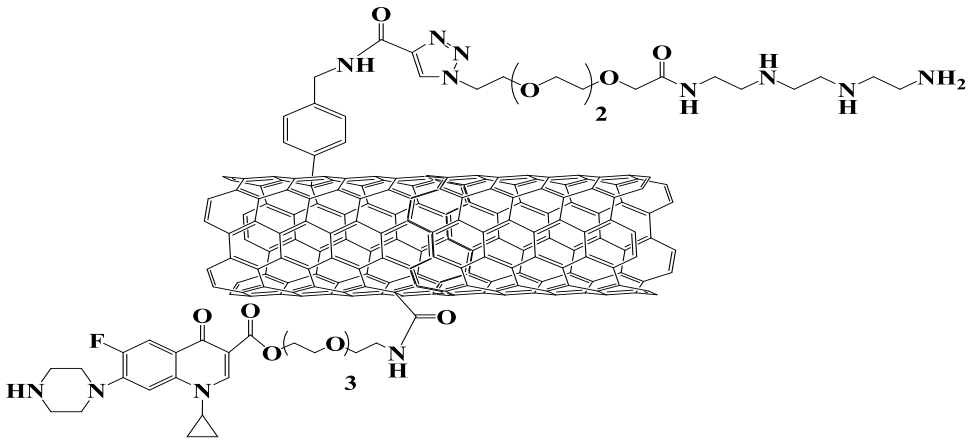
To *f*-SWCNTs **(11)** (18 mg) solubilized in (2.5 ml) of DCM and sonicated for 10 min, TFA (2.5 ml) was added to reaction and stirred for 24 hr. A total of 15 ml of MeOH was added then sonicated and filtered by vacuum. Washing steps were repeated with MeOH (30 ml), and ether (50 ml). The gained Weight of the dried black powder was (16 mg).

2.3.13 Functionalization of *f*-SWCNTs (11) with compound 5 (13)



L-ascorbic acid sodium salt (5 mg, 0.02 mmol) and anhydrous $CuSO_4$ (12 mg, 0.07 mmol) were dissolved in 5 ml of distilled water. The solution was added to a sonicated of *f*-SWCNTs **(11)** (30 mg) and compound **(5)** (97 mg, 0.15 mmol) dissolved in 5 ml of dichloromethane, and the reaction was stirred overnight. Methanol (30 ml) was added to reaction, then sonicated for 15 min then was filtered, product was washed with (2 x 30 ml) MeOH, and (2 x 30 ml) ether. Black powder was dried by vacuum. Then resulting black product was collected and weighed to obtain *f*-SWCNTs **13** (34 mg).

2.3.14 Deprotection of *f*-SWCNTs 13 (14)



f-SWCNTs (**13)** (30 mg) was solubilized in 5 ml of DCM and sonicated for 10 min, TFA (4 ml) was added, reaction was stirred overnight. 30 ml of methanol was added then sonicated and filtered by vacuum. Washing steps were repeated with (2 x 30 ml) MeOH, and (2 x 30 ml) ether to obtain black powder (28 mg).

2.3.15 The Quantitative Kaiser Test protocol [that to determine the free NH_2 loading].

The experiment was done in according to the literature. The result was expressed as mmole of amino groups per gram of SWCNTs [7]. (1.1 mg) was weighted in a small tube. Phenol solution (75 µl), pyridine solution (100 µl) and ninhydrin solution (75 µl) were added to the tube. The blank was prepared exactly with the same quantities of solvents but without the functionalized SWCNTs .The resulting dispersion was sonicated for 5 min. and heated for 10 minutes at 120 ºC. The suspension was cooled and diluted with 60% ethanol (1ml) and filtered by glass dropper. The tube was washed with 60% ethanol (2 x 0.5 ml). After that, the filtrate was analyzed by UV spectroscopy at 570 which indicates the amount of free amine functionalized on the CNTs surface using this equation:

$$\mathbf{NH_2\ Loading\ (mmol/g)} = \frac{\mathbf{[Abs_{sample} - Abs_{blank}] \times dilution\ (mL) \times 10^3}}{\mathbf{Extinction\ coefficient \times sample\ weight\ (mg)}}$$

2.3.16 Adsoprtion of Clarithromycin on *f*-SWCNTs 12 and 14

To *f*-SWCNTs **(12 or 14)** (2 mg) dispersed in 1 ml of carbonate buffer ((Na_2CO_3:$NaHCO_3$) (9:1) at pH 9.16) and sonicated for 5 min, Clarithromycin (2 mg) was added for each sample and sonicated for 30 min. Then it incubated for 24 hr at 25 ºC, and centrifuged for 10 min at 15000 rpm, obtaining *f*-SWCNTs (12-clarithromycin) **(15)** and *f*-SWCNTs (14-clarithromycin) **(16)**

2.4 Preparation of calibration curves

2.4.1.1 Calibration curve of Ciprofloxacin

A calibration curve of Ciprofloxacin at λ_{max}280 nm (doesn't interfere with other λ_{max}) was prepared by using serial dilutions (0.006, 0.005, 0.004, 0.003, 0.002, 0.001, 0.0005 mg/ml) of stock solution of Ciprofloxacin (5 mg/25 ml) in methanol.

2.4.1.2 Calibration curve of clarithromycin

A calibration curve of Clarithromycin at λ_{max} 230 nm (doesn't interfere with other λ_{max}) was prepared by using a serial dilution (0.2, 0.15, 0.1 and 0.05 mg/ml) of stock solution of Clarithromycin (20 mg/10 ml) in dichloromethane.

2.5 Antibacterial activity

2.5.1 Bacterial strains

The antibacterial activity of *f*-SWCNTs **(12), (15)** and **(16)** were studied against *S.aureus* (ATCC 25923), *E.faecalis* (ATCC 700221), *MRSA* and *P.aeruginosa* (ATCC 27583) strains and compared to the activity of Ciprofloxacin and Clarithromycin alone.

2.5.2 Mueller Hinton Broth preparation

Mueller Hinton broth (MHB) was applied to prepare serial dilution of examined substances to detect their antibacterial activity. It was prepared according to the manufacturer.

2.5.3 Bacterial suspension preparation

From fresh bacterial culture, a suspension was prepared with a turbidity equivalent to the 0.5 McFarland standard solutions, which correspond to a concentration of 1.5×10^8 CFU/ml. The prepared bacterial solution diluted to achieve a concentration of about 5×10^7 CFU/ml and the final standard bacterial concentration was set to 5×10^5 CFU/ml in each well [58, 59].

2.5.4 Broth micro dilution method

It is a basic method in this research to determine the MIC (minimum inhibitory concentration). The used protocol was used according to the CLSI [58, 59] *f*-SWCNTs (**12**, **15** and **16**), Ciprofloxacin and Clarithromycin were dissolved in tween 1% to obtain a concentration of 10

µg/ml for each bacterial strain. Then these solutions and suspensions were serially diluted (2-fold) 11 times (in 11 wells) with BBL Mueller Hinton broth (BD, USA) that has the porcine esterase enzyme (15 U/ml). Well number 11 was not inoculated by bacteria and was used as a negative control of bacterial growth but well 12 was free from examined substance and was considered as positive control of bacterial growth. After inoculation of bacterial isolates, the micro tray was incubated at 35 ◦C overnight. Broth microdilution technique for each isolate was made in duplicate. Minimal inhibitory concentration (MIC) was considered the lowest concentration of the drug that inhibit the present of any visible growth in the broth media. Minimum inhibitory concentration can also be determined by measuring the absorbance of wells in each plate at λ_{max} 630 nm.

2.6 Hemolysis assay

Hemolysis test was performed on fresh human blood stabilized with heparin. The test was conducted according to the literature. The human RBCs were centrifuged at 1,000 rpm for 20 min at 4°C and washed five times with PBS (pH =7.4). Then the collected RBCs were diluted with PBS 10 times. Positive and negative controls were prepared by adding 1 mL of water on 0.1 mL of the diluted RBCs (complete hemolysis) and 1 mL of PBS buffer (no hemolysis), respectively. Sample solutions were prepared by adding various concentrations of the nanoantibiotic on the diluted RBCs in duplicate. After that, the mixtures were incubated for 2 and 24 h and centrifuged at 5,000 rpm for 1 min. The absorption at 541 nm was recorded

for each concentration and the hemolysis percentage was calculated according to the following equation:

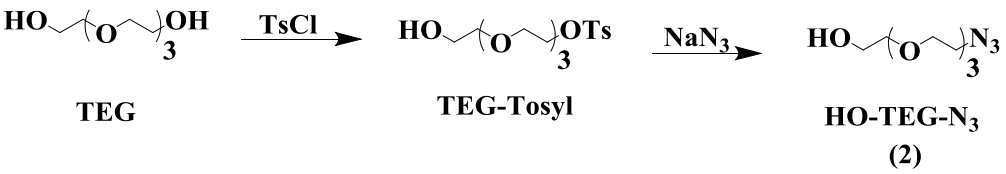
% hemolysis = (Abs of sample -Abs of negative control) / (Abs of positive control - Abs of negative control) ×100

Percentage < 10% is considered safe with no hemolytic activity according to the literature [7].

Chapter Three
Results and Discussion

3.1 Synthesis and functionalization of SWCNTs

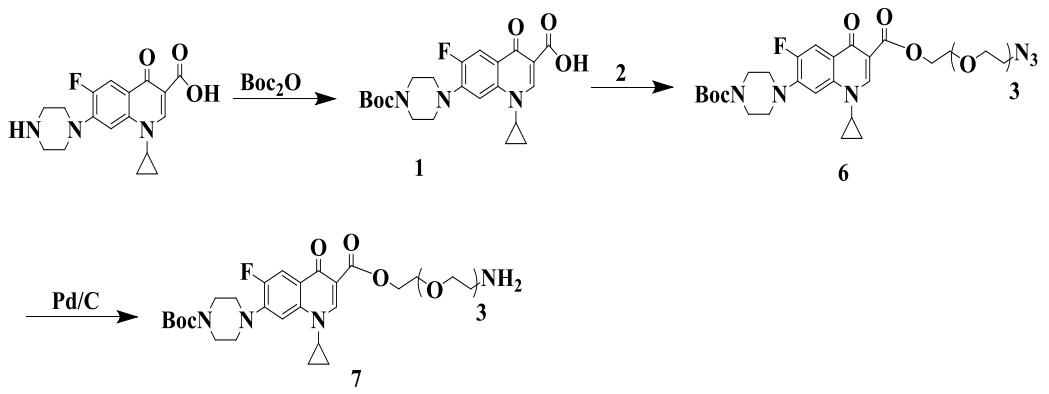
As the main aim of this thesis is the multi functionalization of SWCNTs, various synthetic approaches were used. In first place to attach Ciprofloxacin a hydrolyzable linker was synthesized based on a derivative of tetraethylene glycol started from tosylation of TEG followed by nucleophilic substitution using sodium azide to get OH-TEG-N_3 **(2)**. As shown in Scheme 5.



TEG **TEG-Tosyl** **HO-TEG-N_3 (2)**

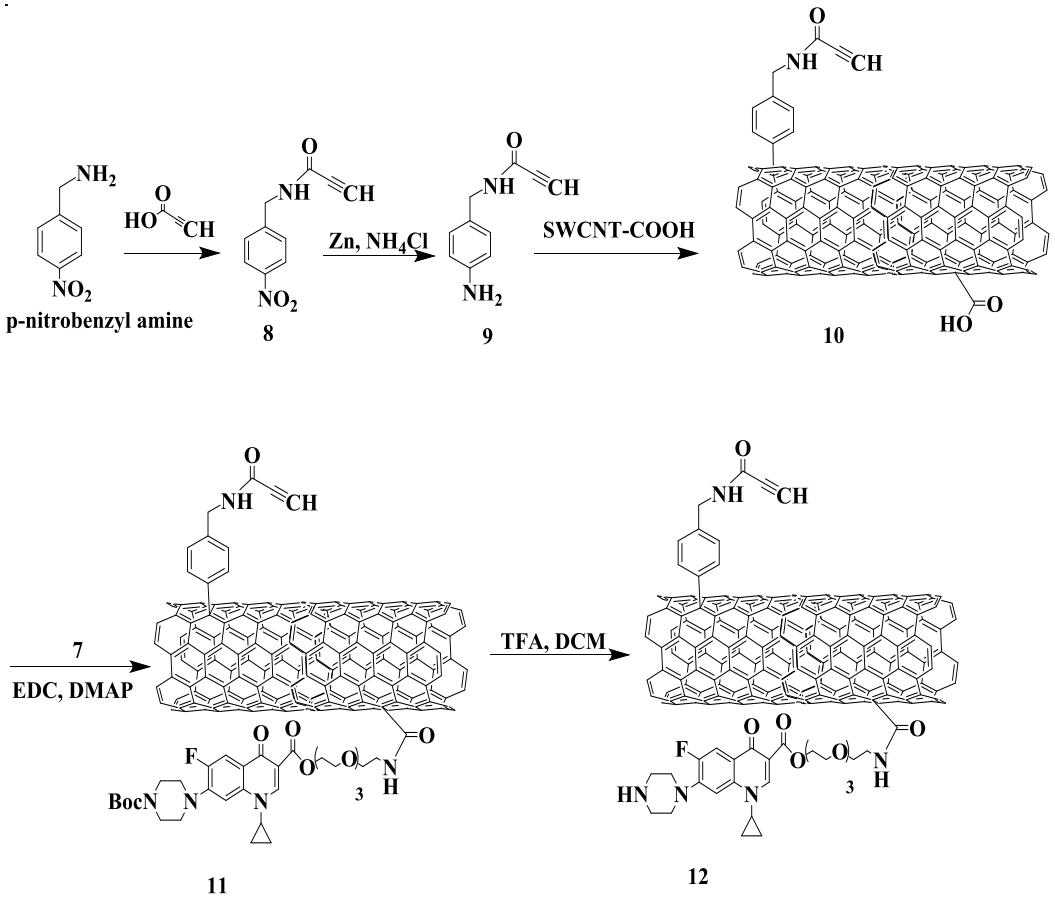
Scheme (5): Synthesis of OH-TEG-N_3.

Then *N*-Boc Ciprofloxacin was reacted with compound **(2)** through esterification reaction using DMAP as catalyst and EDC as coupling agent to achieve compound **(6)**. Product **(6)** was reacted with palladium over carbon with (10%) acidic methanol to reduce *N*-Boc cipro-TEG-N_3 to *N*-Boc cipro-TEG-NH_2 **(7)** as shown in scheme 6.



Scheme (6): Synthesis of N-Boc Cipro-TEG-NH_2.

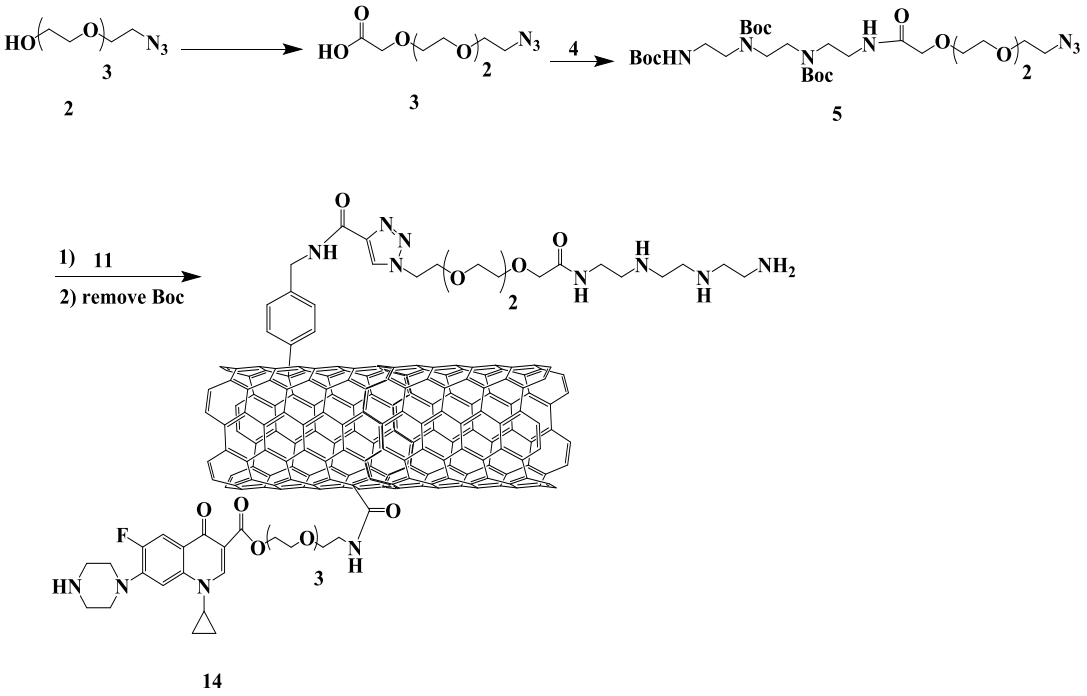
In order to attach compound **(7)** to the SWCNTs, a functionalization of carboxylated SWCNTs was done using Tour reaction**.** p-nitrobenzyl amine was reacted with propiolic acid using DIPEA as Hünig's base and TBTU as a coupling agent to get *N*-(4-nitrobenzyl) propiolamide **(8),** followed by reduction step using ammonium chloride and zinc powder in presence of 10% MeOH to get compound **(9).** SWCNTs-COOH was functionalized covalently using the synthesized product **(9)** in *o*-DCB and CH_3CN. Then an amidation reaction was conducted to attach Ciprofloxacin using EDC as coupling agent and DMAP as catalyst in order to obtain *f*-SWCNTs **(11).** After that, the Boc group was removed from compound **(11)** under acidic conditions using TFA to get SWCNTs functionalized with the Ciprofloxacin as shown in scheme 7.



Scheme (7): Functionalization of alkyne-SWCNTs **(10)** with Ciprofloxacin tetraethylene glycol-NH_2.

In order to improve the penetration to the bacterial cells and improve the antibacterial activity a cationic linker was used. The positively charged molecule will interact through ionic interaction with the cell membrane that has better impact on the antibacterial activity. Therefore, the dual functionalization was achieved by the selective protection of the triethylene tetraamine with Boc groups. The tetraamine protected Boc groups was linker with a derivative of tetraethylene glycol in order to increase the water solubility and facilitate the access to the bacterial cells. Therefore, compound **(2)** was oxidized by Jone's reagent to get compound **(3).** Then, the tetramine-Boc was reacted with linker 3 through amidation reaction

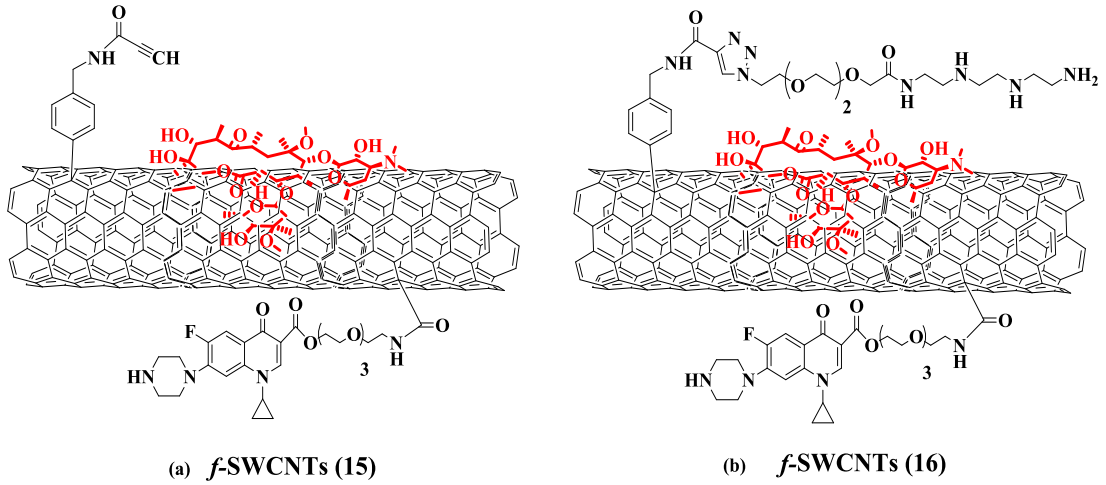
using TBTU as coupling agent and DIPEA as base to get compound **(5).** Then the click reaction was performed between compound **(5)** and *f*-SWCNTs **(11)** to get *f*-SWCNTs **(13)**. After that, the Boc group was removed under acidic conditions using TFA to get the dual functionalized SWCNTs **(14)** as shown in scheme **(7).** The loaded amine value was determined by Kaiser test [60]. The amine loading was 0.05 mmol /gram of *f*-SWCNTs **(14)** as shown scheme 8**.**



Scheme (8): Dual functionalization Alkyne-SWCNTs-Cipro-TEG-NH_2 with triethylenetetraamine oxidized TEG-N_3 **(14).**

Combination therapy is one of the successful approaches that used to improve the pharmacological activity with broader spectrum and also to reduce the bacterial resistance and the required dose. Therefore, in this thesis we aim to adsorb Clarithromycin noncovalently on the surface of the *f*-SWCNTs as it is well known that it has a large surface capacity. So,

Clarithromycin was adsorbed on *f*-SWCNTs **(12)** that have Ciprofloxacin attached to obtain *f*-SWCNTs **(15)** as shown in scheme 9a. In addition it was adsorbed on *f*-SWCNTs **(14)** to obtain triple functionalization with Ciprofloxacin, Clarithromycin and tetramine linker as shown in scheme 9b.



(a) *f*-SWCNTs (15) (b) *f*-SWCNTs (16)

Scheme (9): Non-Covalent functionalization of Clarithromycin with *f*-SWCNTs (12) and (14)

3.2 Characterization of Cipro-SWCNTs

3.2.1 Morphology and size of the functionalized SWCNTs

A microscopic estimation was conducted with transmission electron microscope (TEM) to measure the size and morphology of *f*-SWCNTs **(15).** As it is known, prisitne SWCNTs have low water dispersibility due to the hydrophobic nature of the carbon nanotubes. Moreover, it is found in big bundles due to the Van der Waals interactions between the nanotubes as shown in figure 3.1.A. Therefore, once they are functionalized these carbon nanotubes become more dispersed and the nanotubes will be separated which indicates the successful functionalization as shown in figure 3.1.B.

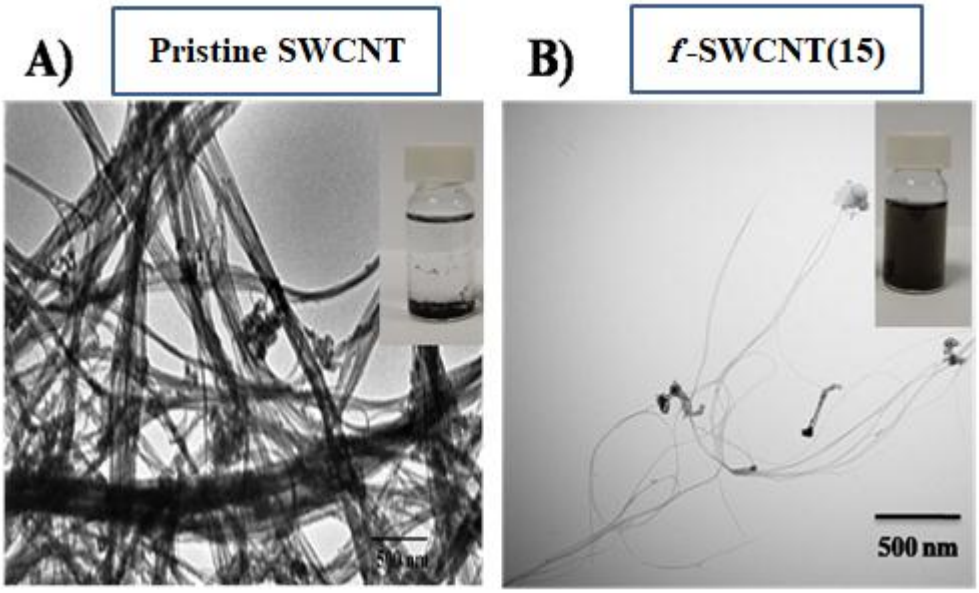


Figure (3.1): TEM image of prisitne SWCNTs with inset of photographic of the p-SWCNTs, B) TEM image of *f*-SWCNTs and inset vial showed the disperesibility of *f*-SWCNTs **15**.

3.2.2 Quantification of Ciprofloxacin and Clarithromycin

The loaded amount of Ciprofloxacin and Clarithromycin were measured by UV-Vis spectrophotometery (blank was pristine SWCNTs). A calibration curves have been constructed at λ_{max}280 nm with a R^2 0.9971 for the Ciprofloxacin and at λ_{max}230 nm with a R^2 0.9983 for the Clarithromcyin as shown in figures 3.2. The loaded amount of Ciprofloxacin on SWCNTs on all cases was about 50 μg/mg and 7 μg/mg of Clarithromycin for *f*-SWCNTs **(15)** and **(16).**

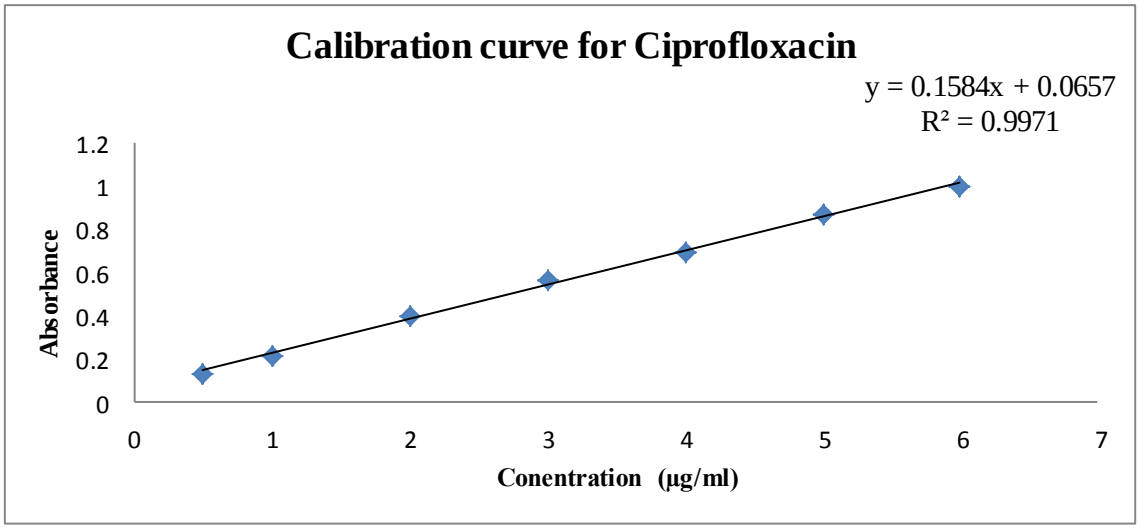


Figure (3.2.A): Calibration curve of Ciprofloxacin in methanol at λ_{max}280nm.

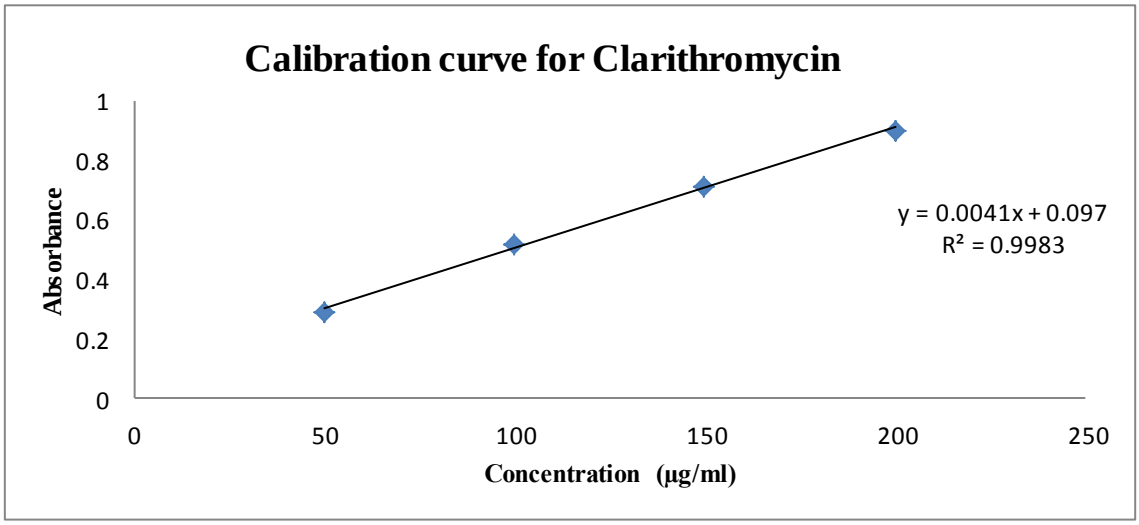


Figure (3.2.B): Calibration curve of Clarithromycin in DCM at λ_{max}230 nm.

3.2.3 Thermogravimetric analysis (TGA)

A thermogravimetric analysis was conducted in order to quantify the total amount of the functionalization on the surface of the SWCNTs in final *f*-SWCNTs **(16).** As the single walled carbon nanotubes are thermostable, therefore upon gradual heating of the sample, a decrease of the sample

weight was observed as the attached molecules on the surface of the SWCNTs will be

degraded. Therefore, we have observed 62% weight loss, which indicates the successful triple functionalization of the SWCNTs as shown in figure 3.3.

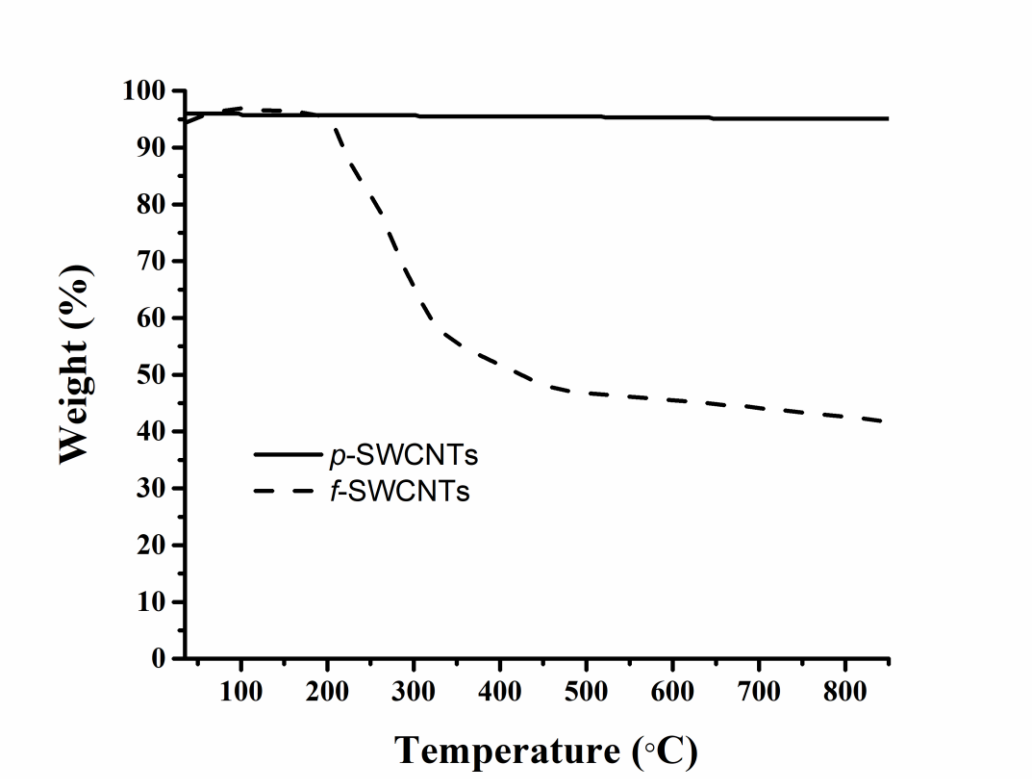


Figure (3.3): TGA analysis of *f*-SWCNTs **(16).**

3.3 Antibacterial activity

3.3.1 Broth microdilution method

The MIC of Ciprofloxacin, Clarithromycin, *f*-SWCNTs **(12),** *f*-SWCNTs **(15)** and *f*-SWCNTs **(16)** were determined by broth microdilution technique on four bacterial species including Gram-positive bacteria

(*Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus* and *Enterococcus faecalis)* and Gram-negative (*Pseudomonas aeruginosa)*. Figure 3.4 shows a representative result of microdilution technique.

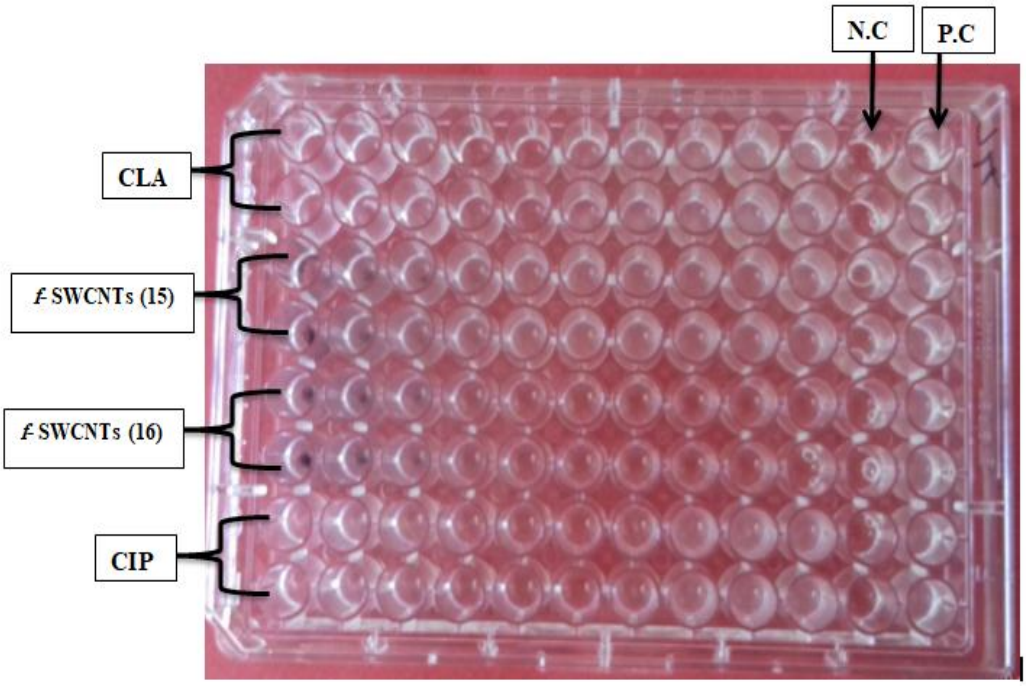


Figure (3.4): Microbroth dilution method for MRSA (first two rows) and *S.aureus* (the remaing six rows).P.C, Positive control; N.C, Negative control; CLA, Clarithromycin; CIP: Ciprofloxacin.

It was observed that Ciprofloxacin has showed antibacterial activity on the four tested bacteria. However, Clarithromycin showed antibacterial effect only on *S.aureus* and *MRSA*. Once the SWCNTs functionalized with the Ciprofloxacin *f*-SWCNTs **(12)**, its antibacterial activity was improved on all four tested bacteria. However, the activity was only enhanced two folds in the case of *MRSA*. Therefore, the multi functionalization was conducted to have a better enhancement on the all tested bacteria. As can be observed in table 3.1, the antibacterial activity of *f*-SWCNTs **(15)** was improved significantly in comparison to the Ciprofloxacin by 31 folds in the case of

S. aureus and *MRSA*. Moreover, the clarithromycin resistant bacteria (*P. aeruginosa* and *E. faecalis)* became sensitive to the multi functionalized SWCNTs **(15)** due to the presence of the Ciprofloxacin with an MIC 0.02 µg/ml for *P. aeruginosa and* 0.04 µg/ml for *E. faecalis*. As shown in table 3.1.

Moreover, we have studied the effect of the tetramine linker on the antibacterial activity. As can be observed *f*-SWCNTs **(16)** showed a big improvement in the antibacterial activity against *P. aeruginosa* with four folds and two folds on the Gram- positive bacteria.

Table (3.1): Antibacterial activities of examined substances

Bacterial species	**Minimum of inhibitory concentration (µg/ml)**			
	S.aureus	***P.aeruginoa***	***E.faecalis***	***MRSA***
Ciprofloxacin	**0.313**	**0.313**	**0.313**	**0.625**
Clarithromycin	**0.16**	**No effect**	**No effect**	**0.313**
***f*-SWCNTs (12)**	**0.02**	**0.02**	**0.04**	**0.313**
***f*-SWCNTs (15)**	**0.01**	**0.02**	**0.04**	**0.02**
***f*-SWCNTs (16)**	**0.005**	**0.005**	**0.02**	**0.01**

It is noticed that the functionalization improved the antibacterial activity compared with antibiotics alone due to the aggregation of bacteria with Single-Walled Carbon nanotubes, and as a result, the exposing of bacteria to antibiotic increased and consequently the concentration of internalization will be increased. Moreover, the synergistic effect was observed in the case of *f*-SWCNTs **(15)** as clarithromycin resistant bacteria became sensitive to

the *f*-SWCNTs **(15)**. This improvement could be explained by the combination therapy effect. In third place, the presence of tetramine linker enhanced the antibacterial as it played an important role to improve the penetration to the bacterial cells and also to reduce the bacterial resistance by the positively charged molecule, which will interact through ionic interaction with the cell membrane that has better impact on the antibacterial activity.

It is observed that tetraamine cationic polymer more responsive to Gram-positive and Gram-negative bacteria, but the stronger effects were observed against *P. aeruginosa* (Gram-negative bacteria) compared with *E.faecalis* (Gram-positive bacterium). It can be intended that the usually lower efficacy against Gram-positive bacteria may be obtained from the differences in membrane structure and the thickness of the peptidoglycan layer. The peptidoglycan layer of the cell wall of Gram-positive bacteria (20–80 nm) is normally thicker than that of Gram-negative ones (7–8 nm). The thicker cell wall structure of *E .faecalis* may hinder the penetration of the molecules in the cell wall [61].

3.4 Hemolysis assay

The hemolysis assay is supposed to be an essential feature for preclinical research. Canapè et al. have described the hemocompatibility of the *f*-SWCNTs in rat and human RBCs. In this study, the hemolytic activity of the produced nano antibiotic was examined. Table 3.2 presents the percentage of hemolysis at 3 concentrations (0.01, 0.005, and 0.001

mg/mL). It was examined after incubation for 2 and 24 hours. Limited hemolytic activity for used concentrations for these times was detected with hemolysis percentage <10% is shown.

Table (3.2.): %Hemolysis activity of *f*-SWCNT (15 &16)

Conc. (µg/ml)	**%Hemolysis *f*-SWCNTs (15)**		**%Hemolysis *f*-SWCNTs (16)**	
	2 hr	**24 hr**	**2 hr**	**24 hr**
10	2.9	5	1.98	2.17
5	1.87	2.5	0.9	1.8
1	1.1	1.5	0.2	0.7

Conclusion

The combination functionalization using covalent functionalization of SWCNTs-COOH with Ciprofloxacin and the adsorption of Clarithromycin on the surface of the SWCNTs have successfully been obtained. Moreover, multiaamine (cationic chain) was included to improve the penetration of the nanosystem and increase antibacterial activity. The functionalization demonstrated good dispersibility of the *f*-SWCNTs **(15)** as confirmed by TEM. The degree of functionalization was 62% for compound (**16)** as confirmed by TGA. The total amine loading was 0.05 mmol/gram. The antibacterial activity showed improvement of the *f*-SWCNTs **(12)**, **(15)** and **(16)** against four strains of bacteria in comparison with the Clarithromycin and Ciprofloxacin alone. Finally, these *f*-SWCNTs possess good biocompatibility as confirmed by hemolysis assay.

Suggestion for future work

1- Study the in vitro release profile of clarithromycin from SWCNTs (16)

2- Determine the antibacterial activity of *f*-SWCNTs (14) against tested bacteria

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كلية الدراسات العليا

التفعيل المتعدد لأنانيب الكربون النانونية أحادية الجدران للنشاط المضاد للبكتيريا

إعداد

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قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

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الملخص

تعتبر الأمراض المعدية أكبر مشكلة يعاني منها الناس في جميع أنحاء العالم. وأدى الاستخدام الواسع للمضادات الحيوية إلى زيادة المقاومة لهذه الأدوية. ولذلك، طور العديد من الباحثين أنظمة جديدة لتوصيل الدواء قد تدعم تقليل الآثار الجانبية والجرعة الفعالة للدواء. وتعتمد أحد أنظمة إيصال الدواء في هذا المجال على تكنولوجيا أنابيب الكربون النانوية. بالاضافة الى ذلك، استخدمت المركبات الكاتيونية كمرشحات واعدة لتقليل تطور المقاومة.

الهدف من هذا البحث هو تطوير نظام نانوني جديد مضاد للبكتيريا يعتمد على التفعيل المتعدد لأنانيب الكربون النانونية أحادية الجدران من خلال ربط دواء السيبروفلوكسيسين ومركب متعدد الأمينات مع انابيب الكربون النانوية أحادية الجدار من خلال رابطة تساهمية وبالإضافة الى ربط دواء الكلاريثروميسن من خلال رابطة غير تساهمية على سطح انابيب الكربون النانوية أحادية الجدار.

وقد تم تحديد التحميل الأميني من خلال تطبيق اختبار كايزر. وأظهر تحليل الدواء النانوني بواسطة المجهر الالكتروني النافذ فصل وتوزع جيد للأنابيب النانونية التي تشير إلى النجاح الوظيفي. بالإضافة الى ذلك، تم تحديد كفاءة التفعيل لأنابيب الكربون النانونية بواسطة جهاز التحليل الحراري الذي أظهر بنسبة 62 % لأنابيب الكربونية في حالة (16) *f*–SWCNTs.

النشاط البكتيري تم تحديده من خلال استخدام تقنية التخفيف المتسلسل عن طريق تحديد تركيز الحد الأدنى للتثبيط البكتيري لأربعة سلالات من البكتيريا. و قد أظهرت النتائج أن التفعيل المتعدد

لأنانبيب الكربونية أحادية الجدار له أكبر تحسن في النشاط البكتيري بحوالي 64 ضعف بالنسبة لثلاثة أنواع بكتيريا (*Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus and Pseudomonas aeruginosa*) و بحوالي 16 ضعف ل *Enterococcus faecalis* بالمقارنة مع السيبروفلوكسيسين وذلك بسبب اضافة رابط متعدد الأمينات في حالة (16) *f*–SWCNTs. بالاضافة الى ذلك أظهر التفعيل الثنائي والمتعدد للأنابيب الكربونية في كل من (15) *f*-SWCNTs و (16) *f*-SWCNTs على قدرة عالية من التوافق مع خلايا الدم على مدى واسع من التركيز.