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# Antibacterial Effects of Nitric Oxide Releasing Silica Nanoparticles

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# Abstract

Antimicrobial materials are essential in many medical applications. In the recent years a lot of research has been dedicated to nitric oxide-releasing nanomaterials and in particular mesoporous particles in order to improve the storage and delivery performances of nitric oxide carriers for a wide range of applications due to their high surface area-to-volume ratio and easy penetration. In this master's project, the principles underlying in this process are first reported with particular attention towards the mesoporous silica nanoparticles. Next, the process dependence on such parameters as size, shape, and porosity of the silica nanoparticles, and then the surface functionalization with NO donors were also taken into consideration. Porous scaffolds hold great potential for wound healing and skin tissue engineering. Over the past couple of decades, nanoparticles (e.g. silica, gold, and silver nanoparticles) have been extensively explored in wound-healing applications as efficient antimicrobial agents and the use of these nanoparticles has raised concerns. Therefore, there is a real need for the development of scaffolds with controlled release of different antimicrobial and anti-inflammatory agents such as nitric oxide. In this project, the efficacy of silica nanoparticle in releasing the nitric oxide in the effective concentration for anti-bacterial effects was investigated. It is supposed that silica nanoparticles as biocompatible scaffolds, enable the incorporation of nitric oxide with programmable release. This approach will allow the creation of customized antimicrobial structures for a broad range of tissue engineering applications, with particular emphasis in wound-healing applications. The incorporation of a uniform, continuous layers of nanoparticles/antimicrobial (NO@Si) agents was verified by FTIR analysis. Scanning Electron microscopy was used to investigate morphological features of the particles. The antimicrobial efficacy of the antimicrobial scaffolds against a broad range of gram positive and gram negative bacterial (e.g. Staphylococcus aureus and Pseudomonas Aeruginosa) was determined on diverse bacteria isolates. Cytotoxicity analyses of the antimicrobial scaffolds toward fibroblast cells was performed using MTT assay of cell viability and proliferation.

# Contents

# 1- Introduction

## 1-1- Nitric Oxide (NO)

Nitric oxide (nitrogen monoxide, NO) is a gas and a diatomic free radical which has an unshared electron. NO is known as one of the smallest and simplest biologically active molecules in mammalian species [1]. Nitric Oxide is synthesized in human body by means of three synthases known as nitric oxide synthase through l-arginine enzyme catabolism and converting the L-arginine to L-citrulline [1-3]. After the first discovery of NO in 1980s as a vasodilatory messenger and as an endothelium-derived relaxing factor (EDRF), researchers started to discover the ways for the NO generation [4]. In 1992 the NO was recognized as "Molecule of the Year" by Science magazine [5]. Later in 1998 the Nobel Prize in Physiology or Medicine was awarded to American Scientists for their discovery "Nitric Oxide as a Signaling Molecule in the Cardiovascular System" [1, 6-8].

NO is considered as a key molecule in several functions and processes; it regulates several physiological functions and it has therapeutic potential. For example, vasodilation, angiogenesis, cardiovascular regulating the vascular tone, respiratory, wound healing, central and peripheral nervous system regulating neuronal communication, neurotransmission, glaucoma and neural degeneration, in cancer cells and in treating infections it acts as an anti-bacterial agent, and many other applications such as penile erection, septic shock, platelet aggregation, gastrointestinal mobility, genitourinary, respiratory, hormone secretion, gene regulation, hemoglobin delivery of oxygen, stem cell proliferation and differentiation, [2, 4, 9-14].

NO has different and extensive reactivity as it has electronic and radical structure that enables for different interactions and reactions with many biomolecular reagents. Since NO is lipophilic, it can cross most physiologic barriers; therefore, it can reach most of the target cells easily [3]. NO can diffuse along a concentration gradient, permitting it

to rapidly move from cell to cell independent of receptors and channels. Because of its high reactivity and short half-life, NO action and biological impact are determined primarily by rate of formation. Furthermore, since NO can be collected by molecules such as myoglobin and hemoglobin, the site of action can be local with respect to where it is produced [11].

This project has a particular attention towards the antibacterial effects of NO. At high concentrations of NO, bacteria-killing effect can be seen. NO can react with oxygen or reactive oxygen intermediates such as superoxide and hydrogen peroxide to form products with highly oxidizing activities. These reactive species can interact with proteins through reactive thiols, amines, heme groups, and other groups. NO reactions can be done with metalloenzymes, resulting in iron depletion or with free thiol groups which result in the inactivation of metabolic enzymes. NO can also target DNA, to cleave the DNA. Moreover, they can also damage cell membranes. These multifactorial damage to bacteria results in cell death [10]. The functions underlying the process of NO effects on bacteria and specially the bacteria presented in wounds will be discussed later in this chapter.

## 1-2- Antibacterial property of NO

As already mentioned, NO has been recognized with many physiological applications in the immune, cardiovascular, and nervous systems. Recently, the antibacterial property of NO has attracted the attention of many researchers. Currently, the increasing rate of antibiotic-resistant bacteria is one of the most challenging public health's problems. The over-usage of antibiotics has led to wide spread resistance and has made the bacteria antibiotic resistant. It can be seen mostly in the strains such as methicillin-resistant Staphylococcus aureus, vancomycin-resistant Enterococcus and multi-drug-resistant Mycobacterium tuberculosis. The wide-spreading of such strains is increasing at a serious rate [15-17].

Different adaptations can cause the resistance, for example, the changing of the structure of a gene at surface receptors which causes a reduction in drug binding, modification or antibiotic molecular targets, and the synthesis and function of transporter systems where aids the antibiotics removal from the cell and enzymatic functions that leads to the drug decomposition or inactivation [18-20]. Conventional

treatments against these resistant strains require intravenous administration of potent antibiotics at high doses [21, 22].

Nitric oxide has a broad antimicrobial activity against pathogens including viruses, bacteria, and fungi. Antimicrobial effect of NO is dependent on its concentration; NO at lower concentrations (<1 μM) functions as a signalling molecule enhancing the activity of immune cells, whereas at high concentrations (>∼1 μM) increases the level of reactive nitrogen species (RNS) and exerts cytotoxic properties against pathogens. However, eukaryotic cells have evolved scavenging mechanisms to neutralize the destructive effects of RNS, while bacteria are susceptible to these damages and can be killed [23-26].

The main antimicrobial strategies of NO result from nitrosative and oxidative mechanisms. As NO is a radical gas, it is unstable in an oxygen environment and therefore reacts with oxygen or superoxide, forming oxygen and nitrogen intermediates and antimicrobial species such as dinitrogen trioxide (N2O3), peroxynitrite (OONO-), nitrogen dioxide (NO2), S-nitrosothiols (RSNO), and dinitrosyl-iron complexes. At high concentrations of NO (>1 μM), these reactive nitrogen oxide species (RNOS) induce oxidative and nitrostaive damages by chemical alteration of DNA, inhibiting DNA repair enzymes, and lipid damages [23-25].

DNA damage caused by NO derivatives occurs by different mechanisms: RNOS can directly interact with DNA structure, or inhibit DNA repair system, or increase the formation of alkylation agents and hydrogen peroxide with genotoxic activities. For instance, dinitrogen trioxide deaminates adenine, cytosine, and guanine on DNA, while peroxynitrite and NO2• result in the breakage of DNA strands. In addition to, RNOS can inhibit DNA alkyl transferases (DNA repair enzymes during alkylation of DNA) by altering the enzymes through interacting with their cysteine residues. RNOS can also react with tyrosine, methionine, tryptophan, and phenylalanine residues of proteins. Another DNA repair enzyme inhibited by RNOS is ribonucleotide reductase. Peroxynitrite and nitrogen dioxide can also lead to lipid damage in bacteria; the former causes lipid peroxidation and membrane damage which is an antimicrobial activity [23, 24, 26].

Further antimicrobial mechanisms of NO derivatives include S-nitrosylation of thiols (e.g. by RSNO, dinitrosyl-thiol-iron complexes, and N2O3) which can alter protein

function in bacteria, depletion of bacterial iron by binding and removal of heme groups of metalloenzymes, disrupting iron-sulfur clusters in the bacterial respiratory chain, and downregulating ferroportin which is the exporter of cellular iron required for bacterial metabolism [24, 26]. It has also been reported that NO represents an antibiofilm function in a dose-dependent manner. Low concentrations of NO can aid dispersal of biofilm, whereas high NO concentrations may lead to the complete eradication of biofilm [23, 25].

Hence, NO at high concentrations generated endogenously by iNOS exhibits a broad-spectrum activity against bacteria (in planktonic or biofilm form) which is essential for limiting wound infections and thus the progression of wound healing towards a normal process. This inherent antimicrobial activity of NO makes it a promising target for clinical use [23, 26].

### 1-2-1- Acute Wound Healing vs. Chronic Wounds

Normal wound healing is a four-stage process including haemostasis, inflammation, proliferation, and tissue remodelling. Immediately after the injury, haemostasis phase commences to stop the bleeding by local vasoconstriction, platelet aggregation, and clot formation [27, 28]. Within 24 hours following the wounding, inflammation is established which requires the migration and infiltration of immune cells including neutrophiles, macrophages, and lymphocytes. Neutrophils and macrophages play an important role in the clearance of wounds by phagocytosis of cell debris, apoptotic cells, and invading pathogens. Moreover, they release antimicrobial compounds such as reactive oxygen species (ROS), proteolytic enzymes, and proinflammatory mediators. Once the resolution of inflammatory phase is accomplished, proliferative phase initiates. At this stage, deposition of a provisional extracellular matrix (ECM) by fibroblasts, angiogenesis, contraction of the wound edges by myofibroblasts, and formation of the granulation tissue lead to the wound closure and restoration of the structure and function of the damaged area. Ultimately, during the remodelling stage, the granulation tissue formed in the proliferative phase matures and the tensile strength of the newly formed tissue is enhanced [23, 29, 30].

However, impairment of any of the phases of the normal wound healing adversely affects the repair process resulting in the progression to chronic and nonhealing wounds which last over 6 weeks. In this case, the inflammatory phase does not undergo

resolution and there are elevated levels of pro-inflammatory mediators. Chronic wounds can arise from several factors including infection, stress, age, hypoxia at the lesion site, or the presence of a predisposed disease such as diabetes, obesity, and systemic inflammatory diseases [23, 29, 31, 32].

### 1-2-2- Wound Infection

Wound infection is the most common cause hindering the wound healing process and increases the morbidity and mortality among patients. Approximately, 17 million people are reported to develop chronic wound infections and annually 550,000 cases die from these infections worldwide. On the one hand, as in the chronic wounds the tissue breakdown is sustained, the damaged tissue can provide an optimal environment for the bacterial growth and additionally the underlying tissues become accessible to the microorganisms. On the other hand, due to the incomplete microbial clearance, bacteria and their endotoxins can lead to the overproduction of pro-inflammatory mediators prolonging the inflammatory stage [23, 29, 31, 33, 34].

A diverse microbiota constitutes the wound infection, mainly including *Staphylococcus spp., Pseudomonas spp., Streptococcus spp., Enterococcus spp., Corynebacterium spp., and Anaerococcus spp.,* with *Staphylococcus aureus, Pseudomonas aeruginosa, and β-hemolytic streptococci* being the most prevalent ones [29, 35]. Moreover, the antimicrobial resistance phenomenon and the emergence of new bacterial strains not responding to the conventional antibiotic therapy, such as methicillin-resistant *Staphylococcus aureus* (MRSA), have worsened the wound treatment failure [34]. The second complication of infective wounds in reducing the wound healing is the formation of biofilm (e.g. biofilms containing *Pseudomonas aeruginosa*). Biofilm is composed of aggregated bacteria interacting as a community and secrete exopolysaccharide matrix (EPS) which in turn protects the bacteria from host immune defence and reduces the effective penetration of antibiotics giving rise to further antibiotic failure in chronic wounds. Hence, to accelerate wound healing process in chronic conditions, there is an urgency to develop new antibacterial agents which can effectively limit the infected wounds [29, 35].

### 1-2-3- The Role of NO in Wound Healing

Nitric oxide (NO) is an endogenously produced gasotransmitter and mediates inflammation, antimicrobial function, angiogenesis, cell proliferation, and collagen formation during the wound healing process. NO is synthesized from L-arginine through the activity of three different isoenzymes which are generally called nitric oxide synthases (NOS). These NOS include endothelial NOS (eNOS) and neuronal NOS (nNOS) which are constitutively expressed in endothelial cells and neurons respectively and generate low levels of NO, and inducible NOS (iNOS) which is only produced during acute inflammatory response stimulated by invading pathogens or pro-inflammatory mediators. It has been demonstrated that NO generated at lower concentrations by eNOS and nNOS is mainly involved in normal physiological homeostasis and anti-inflammatory process, while higher concentration of NO produced by iNOS corresponds with proinflammatory effects and pathogen clearance during wound healing. Therefore, eNOS-derived NO mediates proliferative and maturation phases and iNOS-derived NO becomes relevant during inflammatory phase of wound repair [23, 24, 36].

As mentioned, nitric oxide exerts its critical functions during three major stages of wound healing process including vascular homeostasis, inflammation, and antimicrobial activity. It has been demonstrated that NO is involved in preventing platelet adhesion to vessel wall, enhancing platelet aggregation and clot formation, regulation of pro-inflammatory cytokines, recruitment of keratinocytes, migration and adhesion of neutrophiles to endothelium, migration and proliferation of fibroblasts, recruitment of leukocytes to the injury site, decreasing bacterial load at wound site, and angiogenesis.

During chronic wounds, however, the endogenous generation of NO is diminished which can arise from several factors such as severe local infection, diabetes, and insufficient blood supply giving rise to the impairment of wound repair. Moreover, considering immune cell recruitment effect of NO, due to insufficient levels of NO in chronic wounds, phagocytic cells are found at lower numbers in chronic wounds and the pathogen clearance efficacy is reduced. Hence, exogenous supplementation of NO at the site of injury can be considered as a potential therapeutic strategy for chronic and non-healing wounds [23, 36].

## 1-3- NO donors

The radical nature, uncontrolled manner and toxicity of Nitric oxide gas to human cells prevent the direct application of NO. Because of the limited utility of NO gas in experimental systems and its instability in the presence of oxidants (e.g., oxygen, oxyhemoglobin, and thiols), synthetic compounds that chemically store and release NO in a controlled manner have been developed. Such "NO donors" facilitate the improved understanding of the crucial function of NO in biological applications and may potentially perform as healing agents and treatment for a number of diseases.

Due to the structural diversity of NO donors, the methods to generate NO by each type of compounds is significantly different [37, 38]. Each kind of compound can be chosen according to the demands of each research and investigation as each donor has distinct biochemical properties. All class of NO donors has the potential of being decomposed, oxidized or reduced as of the presence of the nitrogen- or oxygen-bound. However, similar chemical structures usually have a similar NO-releasing mechanism.

There are a number of different NO donors such as N-diazeniumdiolates, nitrosamines, nitrosothiols, and organic nitrites that are used in therapeutic purposes and are effective in antibacterial applications. Among these donors, N-diazeniumdiolate is as an attractive candidate as it can release Nitric Oxide under physiological conditions spontaneously [39]. In this project, a brief introduction of different NO donors is discussed as follow [40-42].

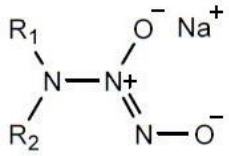
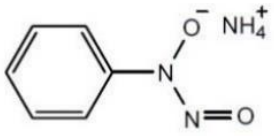
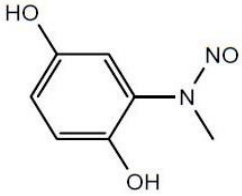
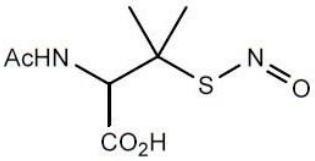
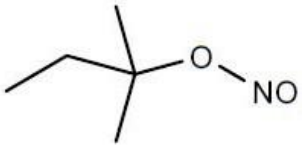
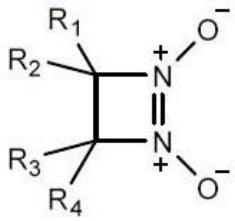
| NO Donor | Structure | Example | NO-release with or without Enzyme |
|---|---|---|---|
| N-Diazeniumdiolate |  | PROLI/NO<br>DETA/NO | Non-enzymatic: Acidic Condition, Heat<br>Enzymatic: - |
| N-Hydroxyl nitrosamine |  | Cupferron,<br>Alanosine | Non-enzymatic: Light, Heat<br>Enzymatic: Peroxidase |
| N-Nitrosamine |  | Dephosatin,<br>N-nitrosourea | Non-enzymatic: Light, Basic Condition<br>Enzymatic: Cyt-P450 related enzymes |
| Nitrosothiols |  | SNAP,<br>S-nitrosogluta-thione | Non-enzymatic: Light, Metal Ions, Thiols, Spontaneous<br>Enzymatic: Unknown Enzymes |
| Organic Nitrite |  | IAN,<br>IBN | Non-enzymatic: Light, Heat, Hydrolysis<br>Enzymatic: Xanthine Oxides |
| Diazetine dioxide |  | 3,3,4,4,-Tetramethyl-1,2-diazetine 1,2-dioxide | Non-enzymatic: Spontaneous, Thiols<br>Enzymatic: Unknown |

Table 1-1. Different kind of NO donors. Abrreviations: PROLI/NO, N-diazeniumdiolated proline; DETA/NO, N-diazeniumdiolated diethylenetriam; SNAP, S-nitroso-N-acetylpenicillamine; IAN, isoamyl nitrite; IBN, isobuyl nitrite.

In this project the main focus is on N-diazeniumdiolate as it is stable under ambient conditions. Most of them are stable at low temperature. N-diazeniumdiolate can be decomposed and generate NO spontaneously in aqueous or physiological solutions. Different rate of NO can be released depends on the temperature and the pH [40].

By reaction of NO with amine groups the NO donors are generated. These reactions can be classified in 2 groups: A) zwitterionic, B) anionic stabilized species. The reactions are presented in Figure 1-1 [39, 42].

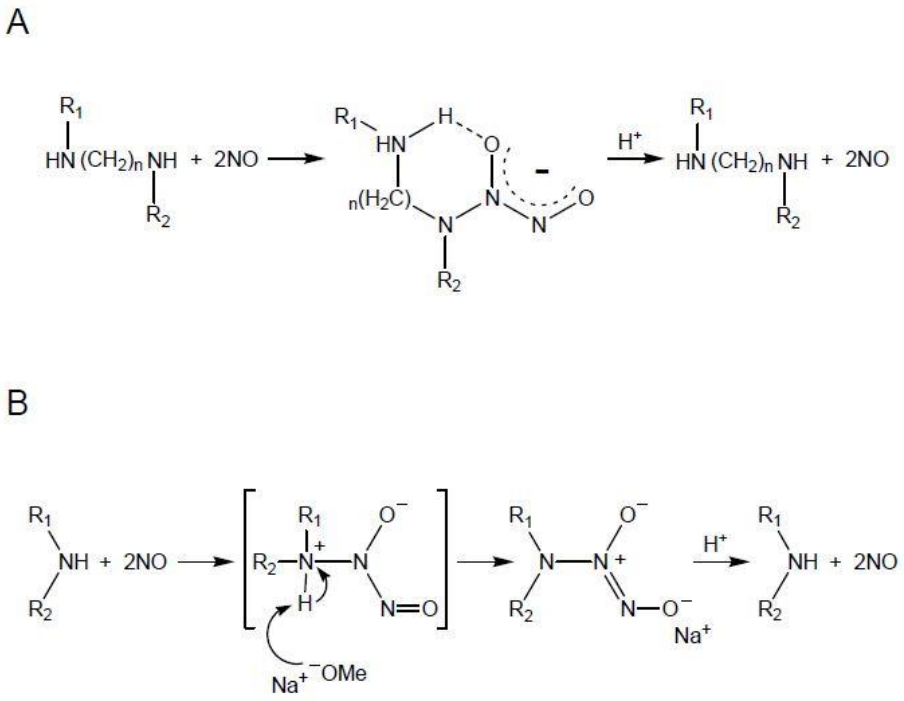



Figure 1-1. Reaction of NO with amine to generate NO donors. A) Zwitterionic B) Anionic Stabilized Species. R1 and R2 are the side groups.

There are different N-diazeniumdiolate with different structures and bonding to amine groups and also with different half-life from 2 seconds up to 20 hours at pH 7.4 and body temperature. Some of the structures are briefly introduced in the Figure 1-2 [43].

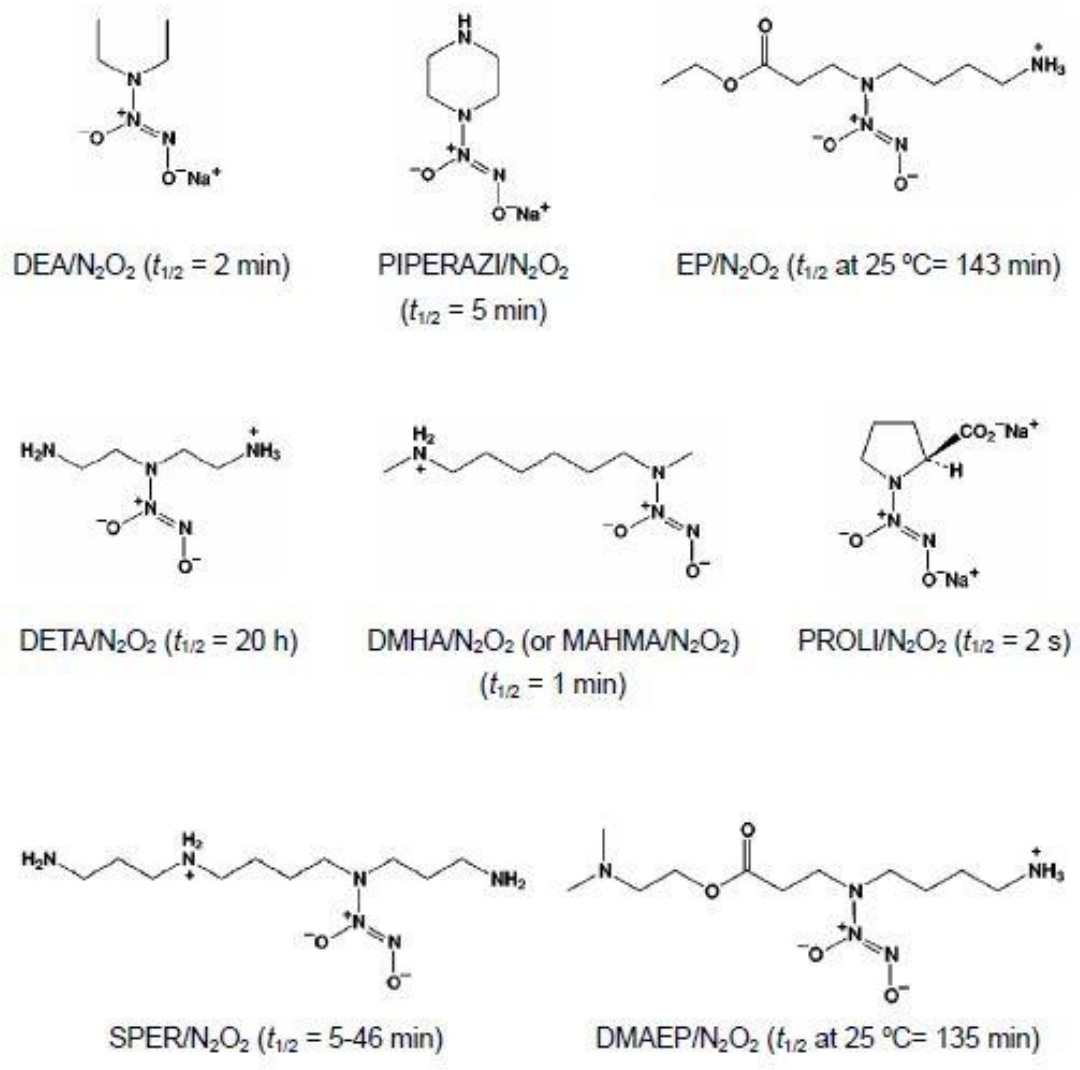



Figure 1-2. Different structure of N-diazeniumdiolates bonded to amine. Abbreviations: DEA, diethylamine; PIPERAZI, piperazine; EP, ethylputreanine; DETA, diethylenetriamine; DMHA, N,N'-dimethyl-1,6-hexanediamine; PROLI, proline; SPER, spermine; and DMAEP, 2-(dimethylamino)ethylputreanine

Although NO donors such as S-nitrosothiols (RSNOs), N-diazeniumdiolate (NONOate) can store and release NO, there are still some limits in their usage as an effective antibacterial agent due to their low storage capacity and uncontrollable release rates. To address this problem, the nanotechnology and nanoparticles are employed in order to increase the storage rate of NO donors which will be resulted in higher release of NO.

## 1-4- NO in Nanotechnology

As already mentioned, the utility of NO is limited because of its short half-life (ranging from 1 second to a few minutes depending on the concentration of oxygen and the presence of NO scavengers such as oxyhemoglobin) [44], instability during storage, and potential toxicity. There is also the lack of methods to be able to control the NO dose and to deliver NO in localized or systemic in vivo. The NO identification as both an antimicrobial agent and angiogenic factor have extended NO research to the field of biomaterials and for the development of NO delivery systems for therapeutic [1, 6, 45]. Nanomaterials are currently being used as a solution to these limitations as they are usually able to store high amounts of NO, they are almost stable, and they have appropriate biological activity. The surface of these materials can be chemically modified and optimized for specific medical applications for example for cardiovascular catheters and its coating where the long and continuous release of NO is required for days or months [13].

Nowadays drug-delivery technologies are used by pharmaceutical companies and about the past two decades, it has been proven that the nanotechnology has a crucial role in drug development and drug-delivery systems' design [46-48] and a progressive increase in the number of commercially available nanotechnology-based drugs [49]. By designing the novel delivery systems the drug side effects and its toxicity to human cells can be reduced, and these systems can be functionalized according to a specific targets [46]. Nanotechnology is considered as a new area and which has promising applications in medicine. Nanoscale drug delivery systems may increase the duration of drug release in the body which can reduce the dose level required for a treatment. Nanomaterials are also able to deliver a drug directly to a target site, reducing its toxicity, which significantly can decrease the side effects [50].

In Nano-systems, at the target site, the higher accumulated of the drug with higher concentrations can be seen due to their small size comparing to the conventional drugs so potentially will be resulted in higher effectiveness of the drug. In addition, the formulation of a drug in a nanoparticulate system can reduce renal and hepatic clearance and decrease immune system recognition [51]. Nanocarriers not only improve the drug solubility and stability, allowing the development of effective compounds that were rejected during preclinical or clinical research due to suboptimal pharmacokinetic or biochemical properties. Thus, nanocarriers ease the development of multifunctional systems for targeted drug-delivery, combined therapies, or systems for simultaneous therapeutic and diagnostic applications. Nanocarriers of nitric oxide make the agent more available to the systemic circulation and also can enhance a target of NO.

Nanomedicine is as an ideal approach to improve the efficiency and wound treatment. Nanoparticles are ultrafine particles between 1–1000 nm in diameter, that have many advantageous properties related to trauma therapy, including being biodegradable, exhibiting controlled drug release, enough drug delivery efficacy, have healing properties, penetrated blood-brain barrier and their ability to be used as an extracellular matrix material. Many studies have shown that nanoparticles can improve antibacterial and wound-healing activities. Specifically, their ability to enhance the penetration of antibacterial agents into deeper areas of the biofilm can improve the efficacy of drugs, avoid inactivation of the biofilm matrix, effectively target infectious cells, and significantly increase the local concentration of antibiotics around bacterial cells [34].

### 1-4-1- NO carriers

The delivery of gaseous NO to target cells like bacteria is challenging due to its high reactivity. Therefore, they high concentration of NO is needed to be effective on target cells. These problems can be addressed by using NO-release scaffolds including inorganic nanoparticles, biopolymeric scaffolds, synthetic polymers, liposomes, etc. [10].

Regarding the antibacterial application of NO these materials can be divided in 2 groups including nanomaterials with intrinsic properties (antimicrobial agent) such as Au, Ag, CNT, Fullerene, ZnO, Chitosan which can enhance the antibacterial effects of NO while NO-releasing into the target cells but they are capable to store a very low concentration

of NO donors in the structure. The other group can be classified as vehicles for delivery such as Silica, PLGA, and Liposome with higher stability and biocompatibility.

## 1-5- Silica nanoparticles

Silica nanoparticles and specially, mesoporous nanoparticles are widely known because of their application in drug delivery and the delivery of other molecules such as proteins or nucleic acid. Some of the advantages of silica nanoparticles as scaffold for NO storage and controlled release of NO are: Stable to pH and T variations, biocompatible, easy to synthesis, tolerability to cells, tuning the size, ability to release NO under irradiation, and the different kinetics of NO release. These advantages made the silica nanoparticles as a widely used materials for NO release [14]. Moreover, post grafting of silica nanoparticles can bind the NO donors such as secondary amines or thiols to the silica structure. Mesoporous silica nanoparticles by having the larger surface area are counted as one of the suitable scaffolds to store NO.

To overcome the limits of low storage of NO donors in other nanocarriers such as Chitosan or Micelles, NO donors can be conjugated to nanocarriers such as Silica nanoparticles or Silica meso porous nanoparticles with the high surface area in order to store a therapeutic dose of NO and subsequently provide a controlled release. Silica Nanoparticles are easy to synthesize with complex structure (core-shell, multilayer, controlled porosity), and they can be functionalized with amine groups through the silanol bond on their surface area. The only limitation of using Silica Nanoparticles is the Silica is not biodegradable. There are some post grafting techniques which are able to convert the solid phase of Silica to liquid which make it soluble in physiological condition.

### 1-5-1- Mesoporous Silica Nanoparticles

Mesoporous silica nanoparticles (MSNs) is considered to be one of the most effective vehicles for NO-releasing because of its large surface area and a high structural order monodispersed pore sizes by using a surfactant. It was reported that the mesoporous silica nanoparticles have extraordinary advantages in comparison to nanomaterials. There are many research interests about the applications of MSNs for biomedical purposes specially the MCM-41 type which has been introduced around 10 years as a drug delivery system [14].

Monodisperse silica spheres were developed 1968 for the first time. The size of particles was ranged from 50 nm to 2000 nm. The Stober process is regularly used for the synthesis of spherical silica particles. In this process, a series of chemical reactions is developed that enables the controlled growth of uniform size spherical silica particles by means of alkyl silicate hydrolysis and subsequent condensation of silicic acid in alcoholic solutions. The morphological catalyst used in this process is Ammonia. One limitation of Stober method is that it provides spherical particles without pores which are not useful to encapsulate organic or inorganic molecules. This problem is resolved by a combined approach between the Stober method and the supramolecular assembly of molecular surfactants to create porous structures. The MCM-41 nanoparticle obtained with this method is porous, however, the pore size is still too small for large organic and inorganic molecules [52].

The synthesis, characterization and application of mesoporous materials have been interesting areas for researcher after the discovery of the M41S materials by the Mobil researchers in the early 1990s. The great and increased surface area, flexible wall-compositions, and finely tuned pore sizes of these materials in mesoporous range has made them interesting for many applications such as catalysis, production of novel materials by encapsulating metals, semiconductors, etc. Mesoporous materials have usually the pore size in the range of 2–50 nm [52].

The synthesis of mesoporous silica is based on the formation of surfactants that serve as templates for the polymerization of orthosilicic acid. The synthesis can be performed either in acidic or basic conditions which will be resulted in different particles diameter, and the source of silica can be fumed silica, sodium silicate, or a tetraethyl orthosilicate (TEOS). Using the higher amount of source for the Silica can give the higher silanol bonds on the surface and make the material as an improved storage scaffold. The first material reported by the Mobil researchers (designated as MCM-41) was micrometer-sized particles with hexagonally ordered mesopores. The morphology of the particles was variable, with a very small amount of hexagonally shaped nanoparticles. Manipulating the reaction parameters resulted in particles with different shapes and sizes. The Stober method of synthesis was first modified by introducing a cationic surfactant as a template to yield a spherical rather than a hexagonal MCM-41 (Mobil Composition of Matter No. 41) structure. They were successful in generating spherical MCM-41 with similar properties as that generated by other methods [53].

### 1-5-2- Sol-Gel Method

Sol–gel method is the most common method for the preparation of silica. This method can be divided into the following four categories: mixing (to form a sol), gelation, aging, and drying. In a typical procedure, alkyl- and organoalkoxysilane precursors are mixed with appropriate amounts of water, methanol or ethanol, and a catalyst (e.g., acid or base), to form a solution (the sol). The silane precursors are hydrolyzed, resulting in the formation of silanol groups (Si–OH). The reactive silanols subsequently cross-react (i.e., condense) with either alkoxy (Si–OR) or other silanol groups to yield siloxane bridges (Si–O–Si) where R is typically a methyl or ethyl group, and R' is an organic functional group.

Eventually, polycondensation reactions lead to the formation of a polymeric gel network. In the following aging and drying processes, polycondensation reactions continue and residual solvent is removed from the interconnected pore network, thereby increasing the strength and density of the gel. Control over the silane precursors and reaction/processing conditions (e.g., pH, solvent, silane/water ratio, catalyst, and drying time and temperature) allows for tremendous physical and chemical flexibility in creating xerogels with tunable porosity, rigidity, and wettability.

Hydrolysis of the alkoxy-group precedes condensation with a neighboring silanol. Hydrolysis and condensation occur simultaneously, in the aqueous alkoxide solution. The sol-gel process is well adapted for composite Nano-powder synthesis, and oxide NPs and as well as for access to organic–inorganic materials.

The sol–gel process allows the synthesis of ceramic materials by means of preparation techniques different from the traditional process of fusion of oxides. High purity and the achievability of uniform nanostructures at low temperatures were the main benefits of sol-gel processing technology. By capping the particles with appropriate ligands, the dispersion can be stabilized in this liquid phase synthesis [54].

The Silicon dioxide has been synthesized by various techniques. There is significant interest in the synthesis of crystalline and uniform material, for the applications in microelectronics, optical, electrical and such various fields. The sol-gel process has been widely shown to be a very flexible route wet chemical techniques for the fabrication of a large variety of photonic materials in various configurations, such as

monoliths, coatings, fibers and films for optical device applications. The metal salt undergoes hydrolysis and poly-condensation reactions to form a gel-like colloidal suspension consisting of both a liquid solvent and a solid metal oxide phase whose morphologies range from discrete particles to continuous polymer networks [55].

# 2- Research Objectives

Among the different applications of NO, this research will mainly focus on the antibacterial properties of NO by means of mesoporous silica nanoparticles. Since the antibacterial effect of NO is dose dependent and at the low concentration, the bacteria-killing effect could not be seen [12], one of the objectives of the research is to improve the NO storage by means of NO donors in the carrier to increase the donors concentration and to reach the effective released amount of NO for bacterial-killing effect. To gain this aim, a surface functionalization (silanization) is needed to link the NO donors such as N-diazeniumdiolate (NONOates) with adequate half-life. The donors are formed by the direct reaction between secondary amine and NO in basic solutions into the carrier. As the amount of NO needs to be sufficient to kill the bacteria and there are different strains of bacteria presented in wound, the release behavior of NO in the broad range of bacteria which are gram-negative and gram-positive are another goal of this research.

# 3- Methodology

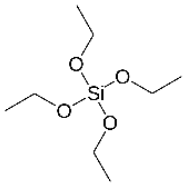
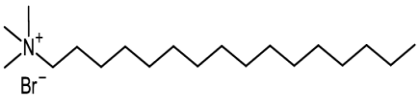
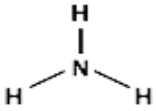
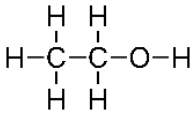
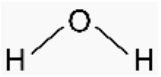
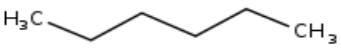
## 3-1- Materials and method to synthesize Silica Nano Particles (SNPs)

Method:

To synthesize the silica nanoparticles (SNPs), the modified Stober method was used by adding a surfactant (CTABr) to produce pores in the structure. This method is resulted in particles with the diameter between 20 to 300 nm according to the amount of ammonia. In a beaker of 600ml, 300ml H2O and 0.6 gr CTABr with 2.25ml NaOH were added under stirring at room temperature. N-hexane was added as a co surfactant in order to increase the diameter of the template to increase the pore diameter. To allow the separation between organic and polar phase the stirring was stopped for 20 minutes. TEOS as a source of Silica was added without stirring and after that the solution was kept for 5 hours under stirring at room temperature. The white precipitate was filtered on a gooch, and washed with water (3 times) and methanol (twice). The white precipitate was dried at 90 C overnight. The powder was calcined in air flow at 550 C for 5 hours to remove the surfactant.

In order to increase the silanol groups on the surface area to increase the post grafting of amine to the particles, the amount of TEOS as the source of Silica was manipulated. The amount of TEOS was increased by considering the amount of solvents and surfactant in order to keep the template to make the pores in the structure.

Table 3-1: List of materials for synthesising silica nanoparticles

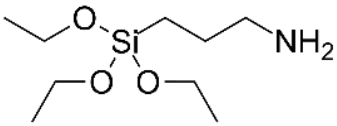
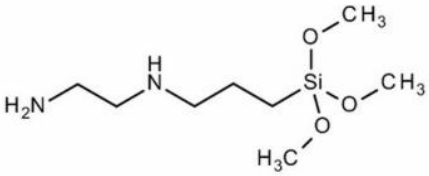
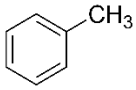
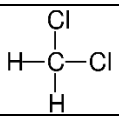
| Material | Formula | Structure | Company |
|---|---|---|---|
| Tetraethyl orthosilicate Tetraethoxysilane (TEOS) | SiC8H20O4 |  | Sigma Aldrich |
| Cetyltrimethylammonium bromide (CTABr) | C19H42BrN |  | VWR Chemicals |
| Ammonia | NH3 |  | Sigma Aldrich |
| Ethanol | C2H6O |  | Fischer Scientific |
| Distilled Water | H2O |  | Nanopure Diamond |
| Hexane | - |  | Sigma Aldrich |

## 3-2- Materials and method for post grafting SNPs with amine functional groups

Method:

In the second phase, amino functionalization was done by adding 1 gr of Silica Nano Particles to 6 gr APTES and AEAP3 in separated solutions. 60ml Toluene was used as a solvent in each sample. The samples were left for 24 hours under stirring till the solvent completely was evaporated. After that the samples were washed three times using dichloromethane and left to dry over night at 80 degree centigrade. The final product grafted with APTES was a white powder. While the final product grafted with AEAP3 was a dense white liquid.

Table 3-2: List of materials for grafting amine group

| Material | Abbreviation | Structure | Company |
|---|---|---|---|
| (3-Aminopropyl) triethoxysilane | APTES |  | Fluorochem |
| N-(2-Aminoethyl)-3-aminopropyltrimethoxysilane | AEAP3 |  | Fluorochem |
| Toluene | - |  | Sigma Aldrich |
| Dichloromethane | - |  | Fisher Scientific |

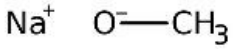
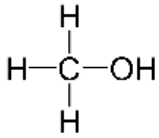
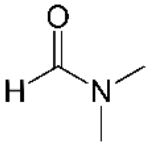
## 3-3- Materials and method for loading Nitric oxide

Method:

The amine functionalized Silica Nano Particles (by APTES and AEAP3) were suspended in 2 ml of MeOH and 18 mmol NaOMe. 18 ml DMF was added to the sample containing APTES. The samples were put in NO reactor bottle, and flushed with Argon gas for 10 minutes and 6 times to make sure that all the oxygen in the suspension were removed. Then the bottle was filled with NO gas up to 5 bar and was kept for 3 days with stirring. After 3 days, the unreacted NO gas was purged from the chamber with Argon gas and its degradation products were removed by using KOH plates.

The samples, containing NONO donors were centrifuged at 5000 rpm for 6 minutes and washed with ethanol twice and were left for drying for 1 hour. The samples were stored at freezer (-20) while sealed until used. The final product was a yellow powder for the sample containing APTES and yellow liquid for the samples containing AEAP3.

Table 3-3: List of materials to load NO on amine grafted silica nanoparticles

| Material | Formula | Structure | Company |
|---|---|---|---|
| Sodium Methoxide (NaOMe) | C2H7NaO2 |  | Alfa Aesar |
| Methanol | CH3OH |  | Fisher Scientific |
| Dimethylformamide (DMF) | C3H7NO |  | Sigma Aldrich |

## 3-4- Materials and methods to control the release of Nitric Oxide

Method:

Before the determination of accumulated amount of NO and its concentration in the solution, a standard calibration curve is required to quantify the $[NO]_2^-$ levels in solutions. The $[NO]_2^-$ is generated by the reaction of NO released from samples with the oxygen dissolved in solution. 250 ml of PBS was used for preparing nitrite solutions with Sodium Nitrite in the range of 1.0 – 100 mmol. 300 µl of the standard solution of given nitrite concentration was combined with 100 µl Griess reagent and 2.6 ml PBS. The 2.0 ml of solution was then incubated for 20 min at room temperature in a dark place. A purple to pink colour appeared immediately. The UV absorbance of the solution with a given concentration was recorded in a scanning range of 200 – 800 nm. The absorbance peak of sodium nitrite and the releases NO is between 510 – 520 nm which is related to the $[NO]_2^-$ concentration. It was observed that by increasing the concentration of Nitrite Sodium solution, the higher peak was appeared in the graph. Therefore, a calibration equation is obtained as follow:

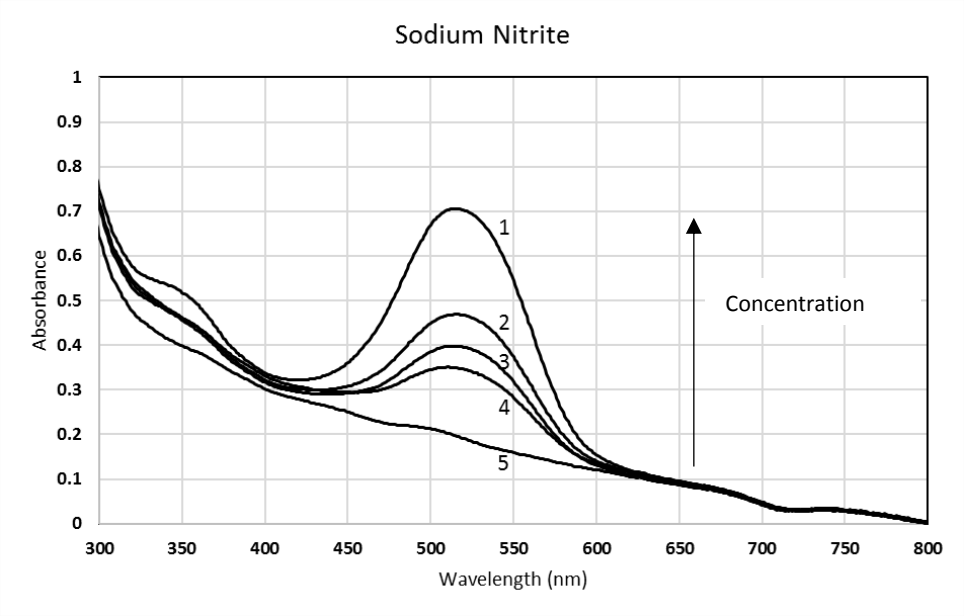



Figure 3-1. UV absorbance at 510 – 520 nm for 4 different concentration of Nitrite Sodium in PBS (No.1-4) No.5 is related to PBS peak

The concentrations were calculated according the following equation considering the volume of 250ml (PBS):

$$C(mM) = \frac{m\ (mg)}{V\ (mL) x\ 69(Nitrite\ Sodium\ molar\ mass)}\ x\ 1000$$

where $m_1$=6.9, $m_2$=3.45, $m_3$=1.72, $m_4$=1.38 mg

Table 3-4. Calibration Curve Calculations

| | C (mmol/L) | ABS | ABS – PBS ABS |
|---|---|---|---|
| Sample 1 | 0.4 | 0.708942 | 0.708942 - 0.202428 = 0.506514 |
| Sample 2 | 0.2 | 0.474537 | 0.474537 - 0.202428 = 0.272109 |
| Sample 3 | 0.1 | 0.372402 | 0.372402 - 0.202428 = 0.169974 |
| Sample 4 | 0.08 | 0.334437 | 0.334437 - 0.202428 = 0.132009 |
| Sample 4 | 0 | 0.202428 | 0.202428 - 0.202428 = 0 |

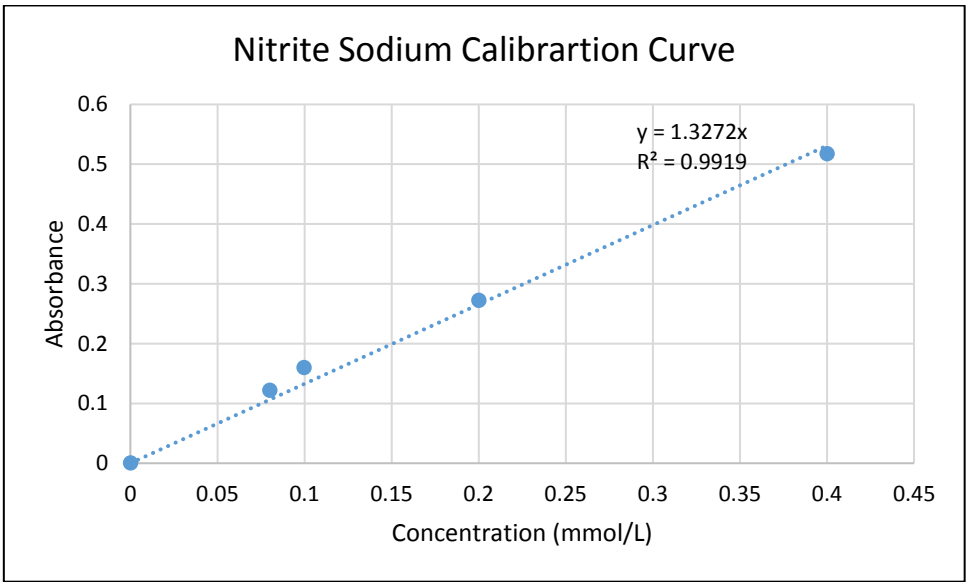
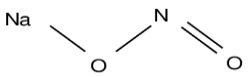



Figure 3-2. The calibration curve of NO concentration and the equation relationship between NO2- and UV absorbance in the Griess test

After calculating the calibration equation, to investigate the NO release, 100 mg of each sample were added in 25 ml PBS. In this step, 4 times dilution was done to evaluate the NO release rate at different concentration for each sample（ratio： 1， 3/4, 1/ 2, ¼). 300 µL of the solution was taken from the solution to mix with 100 µL Griess reagent and 2.6 ml PBS. The 2.0 ml of solution was incubated for 20 min at room temperature in a dark place. The purple to pink colour forms gradually depends on samples concentration. The maximum absorbance is at peak 520 nm on a UV/Vis spectrophotometer. Under the experimental condition, it is assumed the released NO is completely converted into the NO2- in solution. Thus, the calibration equation was used for determination of the NO released from particles.

Table 3-5: list of materials for NO releasing test

| **Material** | **Abbreviation** | **Structure** | **Company** |
|---|---|---|---|
| Griess Reagent | - | - | Sigma Aldrich |
| Phosphate Buffered Saline | PBS | - | Sigma Aldrich |
| Sodium Nitrite | NaNo2 |  | Alfa Aesar |

## 3-5- Materials and method for antibacterial activity

Minimum Inhibitory Concentration (MIC), is a test that determines the lowest concentration of an antimicrobial agent needed to inhibit the visible in-vitro growth of a microorganism (bacteria). The MIC test is done according to PHARM316-PRO-077(V1) protocol, school of Pharmacy, with the following procedure:

At first the nanoparticles were prepared in four different concentration (same ratio mentioned in previous step). Each dilution was poured into a sterile petri dish and plate 50µl using P200 multi-channel pipette into the required wells of a 96 well microliter pipette. Bacteria colony was added to broth to reach an OD equivalent to 1×108 CFU/ml and dilute 1 in 100 (0.1ml OD adjusted culture in 9.9ml fresh medium) to achieve 1×106 CFU/ml. then 50 µl of bacterial suspension was added to each well and mix by pipetting a minimum of 5 times (1 in 2 dilution giving final inoculum concentration 5×105 CFU/ml). Growth control wells with no nanoparticles added should be included; negative control wells with broth and no bacteria should also be included. The minimum inhibitory concentration (MIC) is the well with the lowest concentration of antimicrobial in which no growth is visible to the naked eye. To determine the minimum bactericidal concentration (MBC) plate (in triplicate) 10 µl from the wells one concentration below the MIC and every concentration above the MIC up to the highest concentration onto a suitable agar.

Growth Curves, or anti-bacterial time killing assay was done to evaluate the bacteria killing effects of the nanoparticles in different time periods. The assay was done according to PHARM316 PRO-066 V2 protocol, Pharmacy school with the following procedures:

As mentioned earlier the nanoparticles are prepared in 4 different concentrations. Different isolates of *Pseudomonas aeruginosa* (gram negative) and *Staphylococcus aureus* (gram positive) bacteria are selected to be tested in this assay. All bacteria were streaked from frozen stock onto pre-incubated Mueller-Hinton agar (MHA) plates and incubated overnight under aerobic conditions at 37°C. The bacteria culture (small colony) is added to 20ml broth and is measured until an OD $2x10^8$ CFU/ml is obtained. 12.5 microliter of bacteria as added to each sample (containing 2 ml PBS and 3 ml broth). On T=0 and every 2 hours, 100 micro-liter of each sample is taken out and poured into 900 microliter PBS and diluted 6 times. 10 microliter of each dilution

replicates onto MHA plates for 4 times. The control sample without nanoparticles is also evaluated simultaneously.

To calculate the results, after 24 hours incubation, the total number of colonies at the dilution that gives between 10 and 30 colonies per 10 micro-liter is counted. The following formula is used to calculate the bacteria growth:

$$(\text{AVERAGE BACTERIAL COLONIES} \times 100) \times 10^{(number\ of\ diluation)} = \quad \text{(CFU/ml)}$$

Table 3-6: List of materials for bacteria time killing assay

| **Material/ bacteria** |
|---|
| *Pseudomonas aeruginosa* |
| *Staphylococcus aureus* |
| Mueller-Hinton agar |
| Mueller-Hinton broth |
| Phosphate buffered saline |

## 3-6- Materials and method for Cell Viability MTT assay (Toxicity test)

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye to formazan, which has a purple colour. This assay can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity of potential medicinal agents and toxic materials. MTT is reduced to purple formazan in living cells. A solubilization solution is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The degree of light absorption is dependent on the degree of formazan concentration accumulated inside the cell and on the cell surface. The greater the formazan concentration, the deeper the purple colour and thus the higher the absorbance.

Method:

The MTT assay was done using the 3T3-L1 fibroblast cells of Embryo Tissue. The cells were cultured at Biology school, Queen's University of Belfast using L-Glutamine 200mM, Fetal Bovine Serum, and DMEM + GlutaMAX in Termo Scientific Nunc EasYFlask. The media was changed for 2 days in order to enhance the cell proliferation and achieve an appropriate cell concentration. The final seeded live cell concentration was 3.6 x $10^5$ /ml, and used for the MTT assay at passage number 6. The 2 samples (NO@Si) with APTES and AEAP3, each with 4 gradient concentration starting from 4 mg/mL, 3 mg/mL, 2 mg/mL, and 1 mg/mL (in PBS) were made up in media and added to the cells. 100 µl/well of media + samples in triplicate were added in 3 plates. The plates were incubated for 48 hrs. The supernatant then was discarded and the cells washed once with PBS. 50 µl of thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) solution (2 mg/ml stock in PBS diluted 1:4 in assay media) was added to each well and the cells incubated for 4 hours. Viable cells convert the soluble yellow MTT to insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. The supernatant once again was removed and a 200 µl solution of DMSO added to dissolve the formazan crystals and the plate incubated at

37°C with agitation for 10 minutes. Optical density was then measured at 570 nm with a reference filter at 630 nm.

Viability is calculated as the % absorbance of the sample when compared with the absorbance of the untreated control. The assay was done in the dark since the MTT reagent is sensitive to light.

## 3-7- Characterization

In order to measure the diameter of the nanoparticles and to observe the particles' morphology the Scanning Electron Microscopy (SEM) is done. To characterize the nanoparticles in each step and to define the molecular bands Fourier-transform infrared spectroscopy (FTIR) is done. To measure the surface area and define the type of nanoparticles in terms of size, BET surface area was done.

### 3-7-1- SEM

The scanning electron microscope (SEM) is a technique that allows sample observation with magnification and resolution 1000 times greater than ordinary optical microscopy. The electronic microscope does not use light as a source of radiation, but it exploits an electron beam that is thermionically emitted from an electron gun fitted with a tungsten filament cathode. The signals used by a SEM to produce an image result from interactions of the electron beam with atoms at various depths within the sample. The signals that derive from electron-sample interactions reveal information about the compound including external morphology, chemical composition, crystalline structure and orientation of materials making up the sample. To prepare the samples for SEM, it is important to have conductive samples to receive the scans with high resolution. If the samples are not conductive, a gold coated is used to make a thin layer of gold on the samples in order to make them conductive. Since the silica nanoparticles are not conductive, the coating is done before scanning the samples. The SEM images in this project were taken at Physics school, with field emission scanning electron microscope JEOL JSM-6500F with vacuum $10^{-6}$.

### 3-7-2- FTIR

Fourier-transform infrared (FTIR) spectra is used for surface characterization of nanoparticles using an Agilent Technologies Cary 630 FTIR spectrometer in the range of 650 – 4000 cm-1. FT-IR patterns present vibrational bands which correspond to characteristic organic groups and inorganic bands within a sample. This characterization method is used to characterise synthesised samples to ensure completion of conversion or to show presence of impurities such as unreacted reagents and possible side products. It can also be used after NO loading into amine modified Silica nanoparticles to confirm incorporation of NO donors within the structure which will be resulted in new peaks or shifts in comparison to the samples without NO donors.

### 3-7-3- BET Surface Area

This analytical technique allows the study and the evaluation of surface area, volume, dimension and distribution of pores of materials in observation. These evaluations can be obtained by studying the adsorption of a gas or a liquid on a sample.

In case of gas adsorption, analyzed material is called adsorbent while the gas is named adsorbate. It is possible to distinguish 2 types of adsorption: physical (physisorption) and chemical (chemisorption). The technique used to characterize samples belong to the first class, in which the interaction between adsorbent and adsorbate are weak and can be reconducted to Van Der Waals forces. The most used gas for this kind of analysis is nitrogen (N2) thanks to its low reactivity and low cost. The BET surface area was done at Chemistry School facility, Queen’s University of Belfast.

# 4– Results

## 4-1- SEM

As it can be observed in the figure 4-1-, the silica nanoparticles synthesized by modified Stober method, have the spherical morphology and all the particles are formed in about same diameters which is about 70-80 nano meters. This can be resulted in homogenous post grafting of amine functional groups and then NO donors which could be effective in the release rate of NO. after manipulating different amount of Silica source (TEOS) in the synthesis procedure, we reached the samples with diameter varying from 70 to 270 nm. The sample in Figure 4-1. was selected as the best sample with the highest surface area. The pores in MCM-41 type of mesoporous Silica nanoparticles have the diameter between 2 – 10 nm. Due to small size of the pores we could not observe the pore structure in the SEM images.

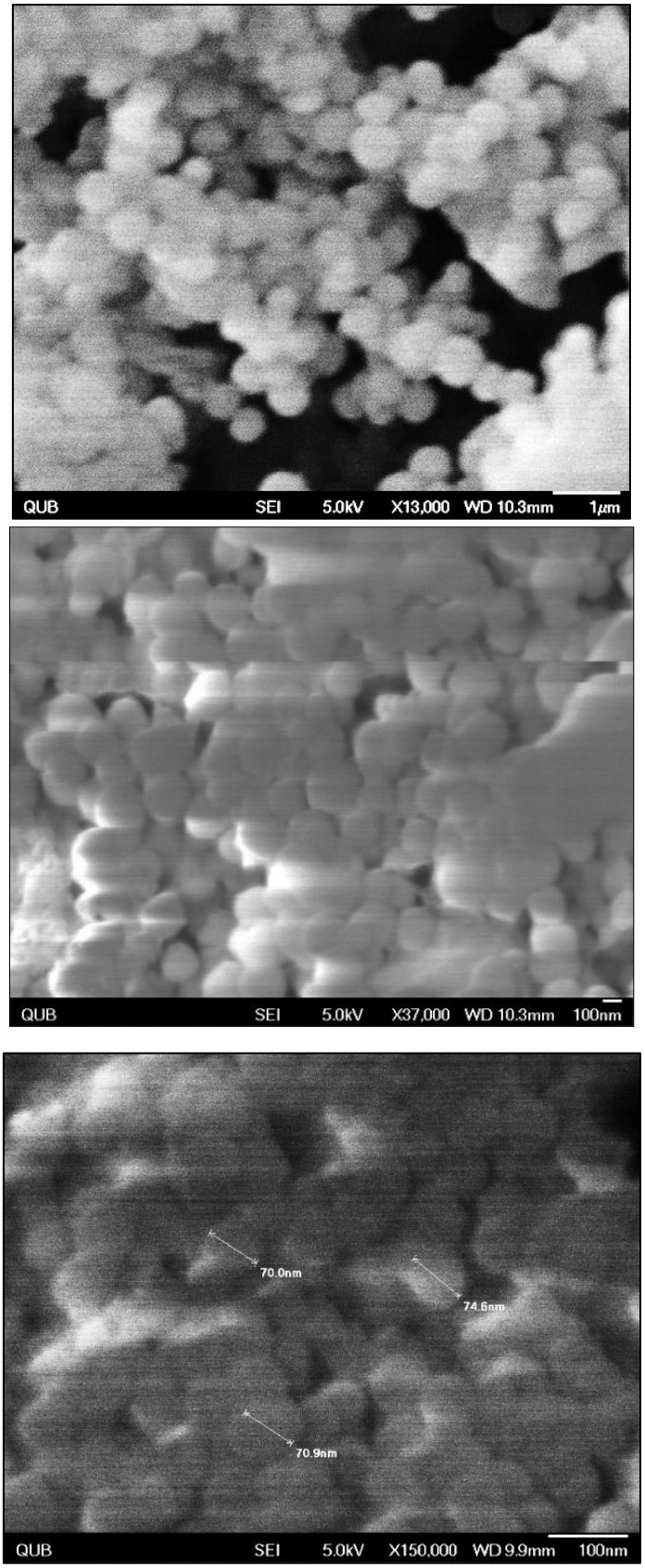



Figure 4-1. SEM images of Silica Nanoparticles in different magnifications

Figure 4-2- shows the APTES grafted to the silica nanoparticles' surface. It can be seen that a layer is formed around the particles which prepare the saurface for Nitric Oxide to react with the amine groups on the surface and to form the NO donors. There is an increase in partciles diameter due to the amine groups (APTES) around the silica nanoparticles.

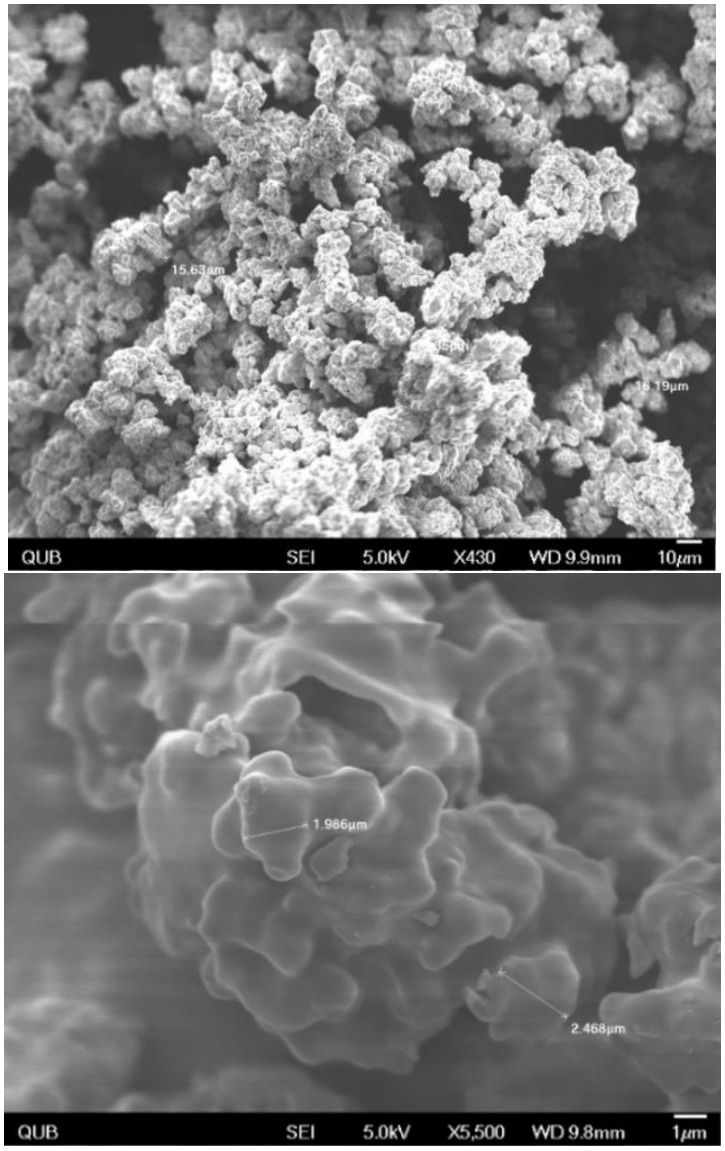



Figure 4-2. SEM images of amine functionalized silica nanoparticles (by APTES)

## 4-2- FTIR

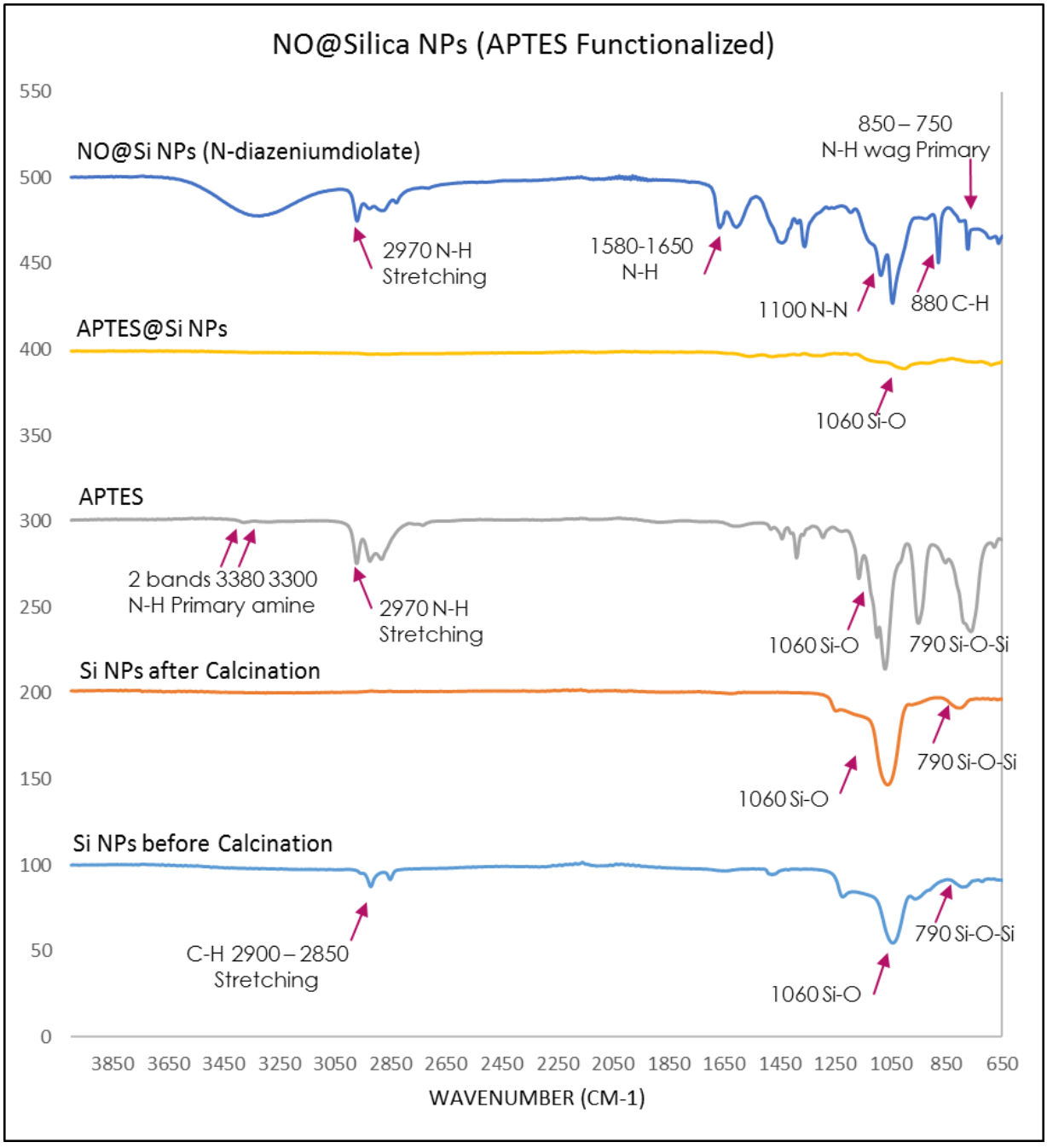



Figure 4-3. FTIR spectra of all steps to prepare NO@silica NPs (APTES)

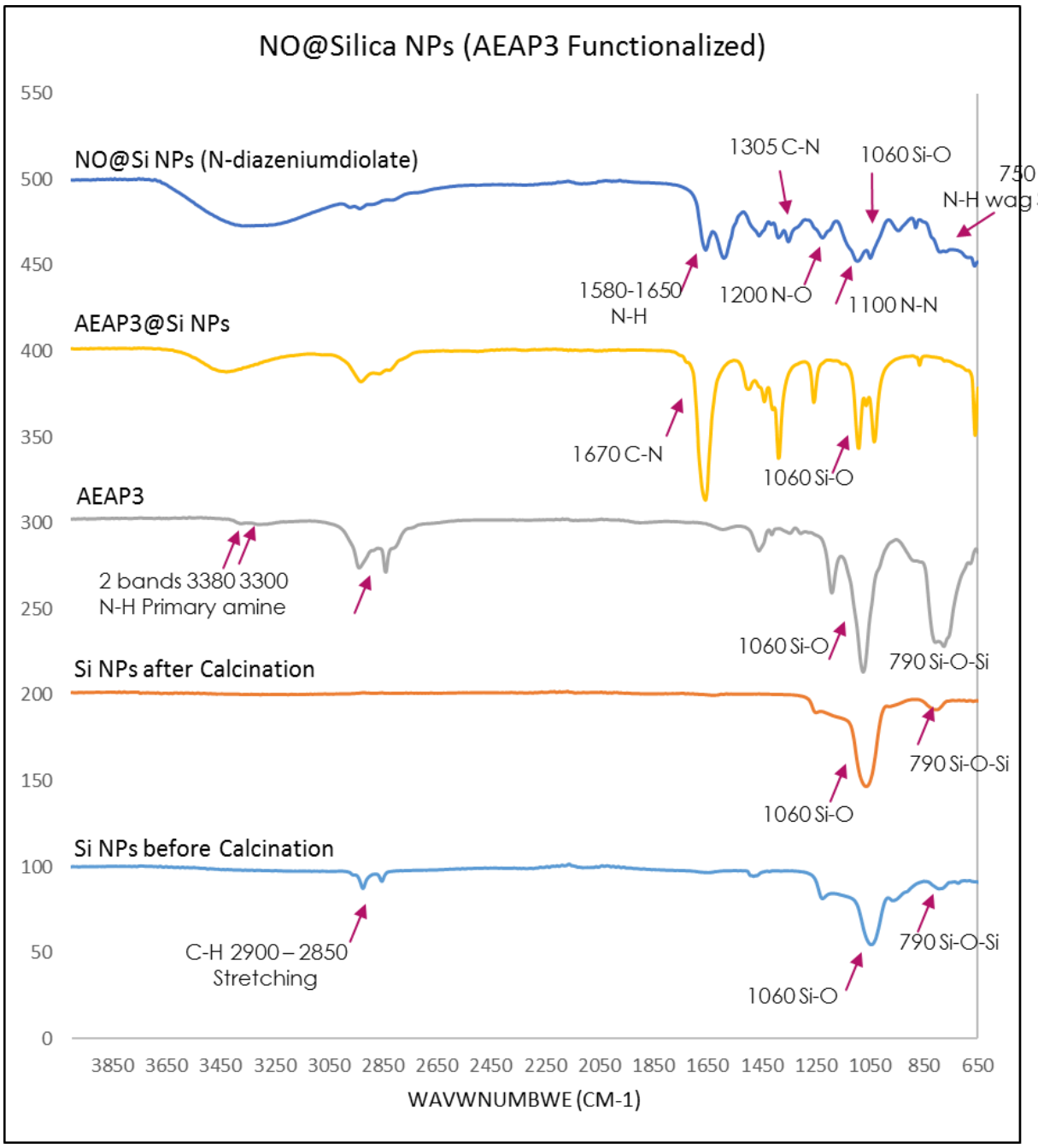



Figure 4-4. FTIR spectra of all steps to prepare NO@silica NPs (AEAP3)

Observing the IR spectra in Figure 4-3. and 4-4. it can be noticed that both calcined SNPs and not calcined SNPs samples show a “silica region” between 400 and 1250 cm-1, in which can be distinguished a band made of: Si-O-Si symmetric bending (458 cm-1), Si-OH rocking (574 cm-1), symmetric stretching Si-O-Si (803 cm-1), symmetric stretching Si-OH (962 cm-1) and asymmetric stretching vibrations Si-O-Si (1061 and 1220 cm-1)[56]

Not calcined SNPs exhibits the typical bands of C-H stretching just below 3000 cm-1 (2924 and 2850 cm-1), and also the peak at 1476 cm-1 due to the C-H bending. On the other hand, calcined SNPs do not provide these bands confirming the fact that the calcination process led to the desired results.

After loading NO to the samples, it can be proved that the bonds related to N-N and N-O has been formed according to the present peaks in the range of 1200-1100 cm-1. There are peaks related to N-H bonds in both samples which shows that both primary amine and secondary amines are presented in the structure which should be due to the presence of both primary and secondary group in AEAP3 structure. And also, the conversion of some of primary amines in APTES to secondary amine by adding DMF to the solution. By this graph, the formation of N-diazeniumdiolate was proved which will be decomposed in PBS and will release NO.

## 4-3- BET Surface Area

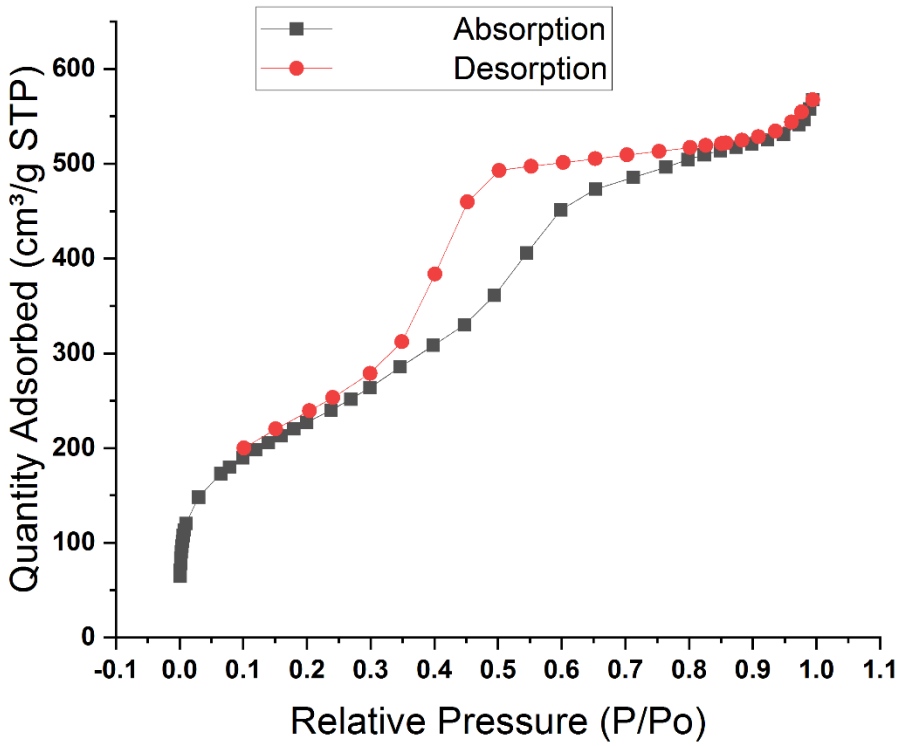



Figure 4-5. Isotherm of Silica Nanoparticles

The isotherm obtained from the BET characterization can be traced back to the typical Type IV thanks to the presence of the hysteresis between the adsorption and desorption branch. This profile proves that the type of Silica Nanoparticles synthesized in this project is mesoporous. The BET surface area reported for this sample was 828.2951 m2/g which proves that there are some pores in the structure in comparison to non-porous silica nanoparticles which has the BET surface area around 300-400 m2/g.

## 4-4- NO release profiles

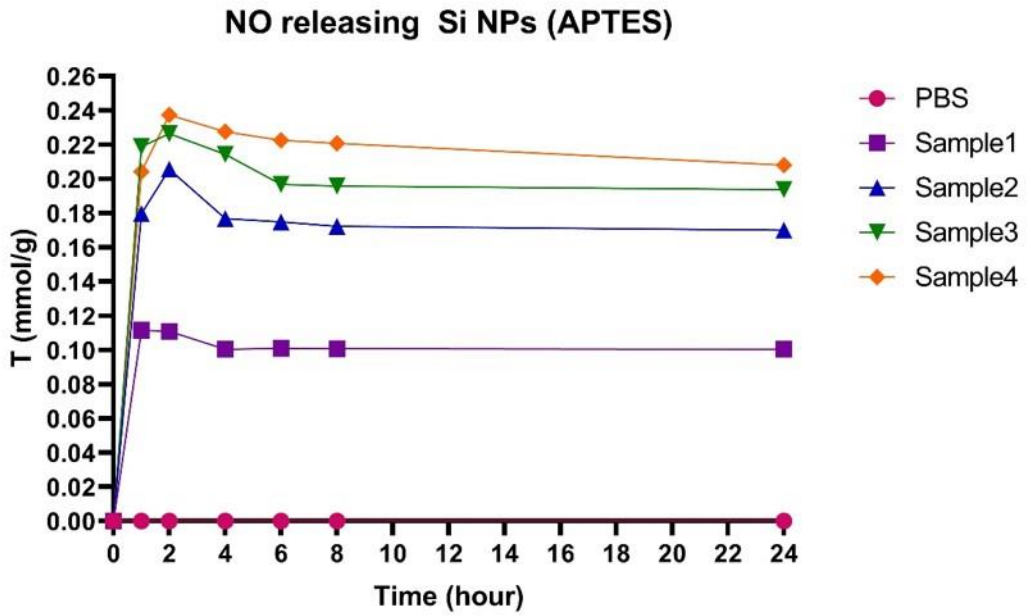



Figure 4-6. No release rate from APTES grafted SNPs

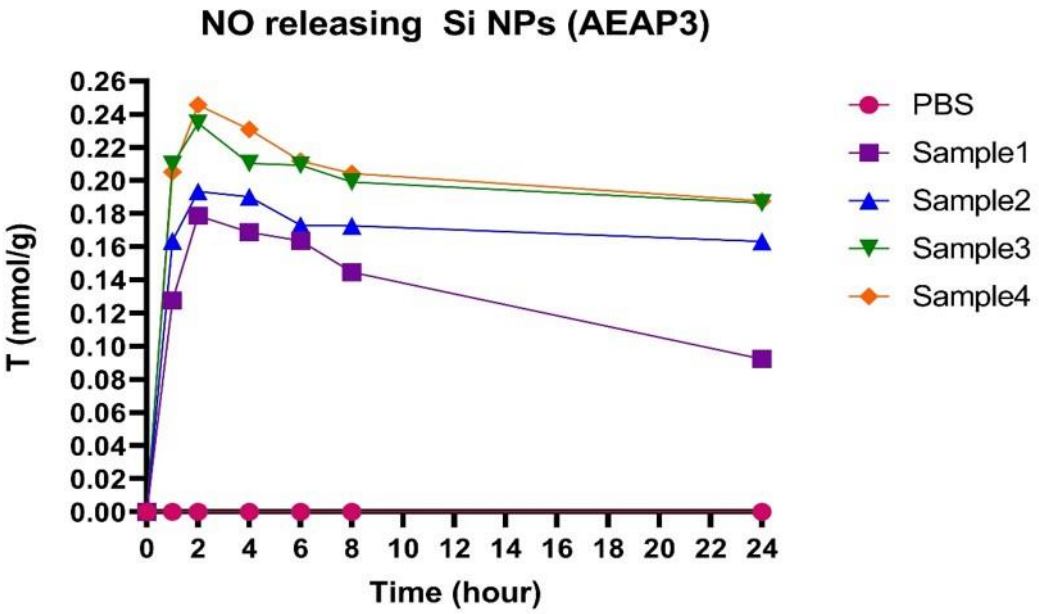


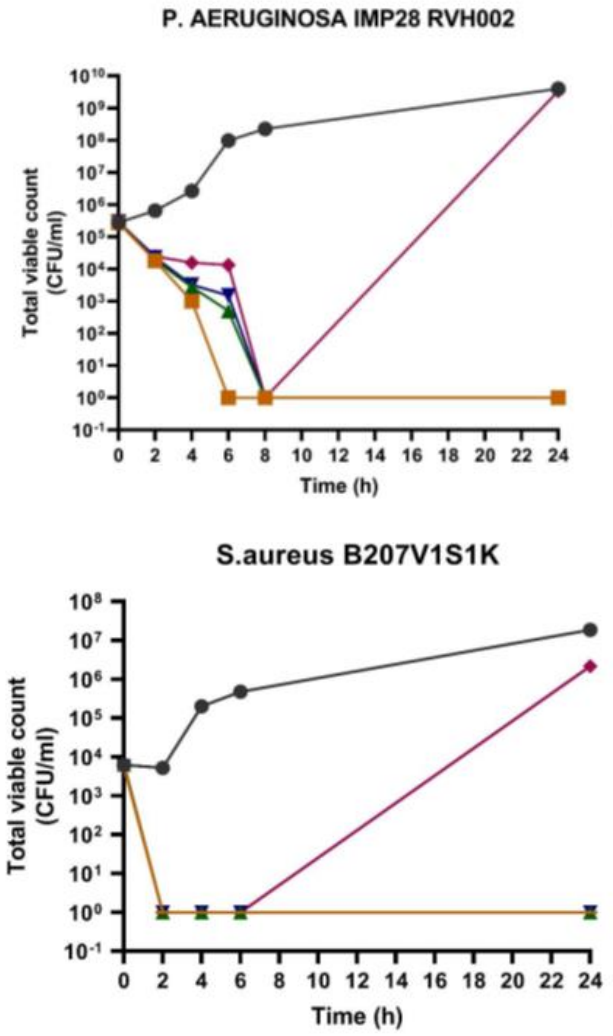

Figure 4-7. No release rate from APTES grafted SNPs

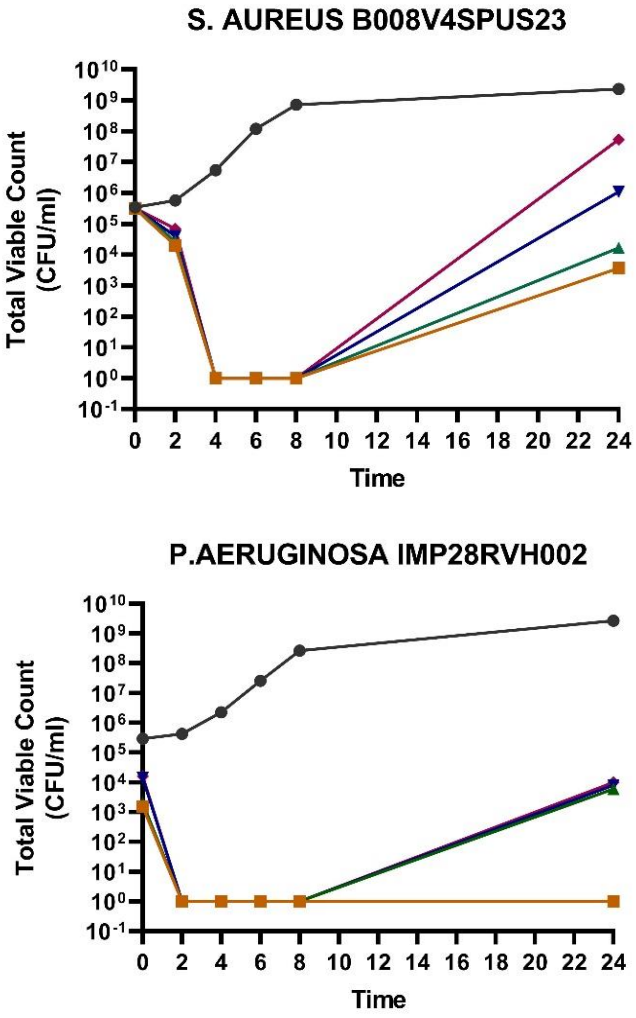
Figure 4-6. and 4-7. demonstrate the release rate of NO as accumulated amount of NO in mmol/gr in time intervals of 2 hours. As mentioned before, each sample were tested with four different concentration. It can be understood from the graphs that by increasing the concentration of samples in PBS the accumulated amount of NO is increased. The highest release is in time period of 2 to 6 hours which is predicted to show the highest anti-bacterial efficacy in this period. The sample containing AEAP3 has slightly more capability for NO storage and in the sample with highest concentration it is capable to store up to 0.25 mmol/gr.

## 4-5- Anti-bacterial killing assay


Concentration: Sample1) 1mg/ml, sample2) 2mg/ml, sample) 3mg/ml, sample4) 4mg/ml

Figure 4-8. Time killing assay of NO@SNPs samples containing APTES on gram-negative (P.AER) and gram-positive (S.AUR) bacteria






Figue 4-9. Time killing assay of NO@SNPs samples containing AEAP3 on gram-negative (P.AER) and gram-positive (S.AUR) bacteria

According to the figure 4-8. and 4-9., it can be observed that the NO releasing Silica nanoparticles are more effective on gram negative bacteria and in higher concentration of each samples. The samples containing APTES, has more effective bacteria killing in gram-positive bacteria which will be dure to higher amount of secondary amine formed after interacting with NO gas (all the primary amines were converted to secondary) while in AEAP3 as there are both primary and secondary amine in the structure, after interacting with NO, the conversion will be different and could be resulted in formation of lower amount of secondary amine which is themost stable and effective amine groups in N-diazeniumdiolate structure as the NO donors. In this study different strain of bacteria were examined and it can be proved that each strain has different result after being treated by NO.

## 4-6- MTT Assay

Exposures were carried out in triplicate wells and three independent exposures were performed (n=3). Data was processed with Excel and Graphpad PRISM software version 9.0 (San Diego, CA). All values shown are expressed as mean ± standard error of mean (SEM) of three independent exposures for the compounds tested. Data is expressed as a percentage of the relevant solvent control for each parameter and analysed by one-way ANOVA followed by Tukey's test for multiple comparisons; the mean concentrations were tested for significant difference at the 95% confidence level. As it can be observed in Figure 4-9. there is no significant difference between control and treated groups ($p>0.05$) which can prove that the NO releasing silica nanoparticles grafted with both amine sources (APTES, AEAP3) at the different concentrations with antibacterial activity (1 mg/ml – 4 mg/ml) had no significant toxicity in Fibroblast cells (3T3-L1).

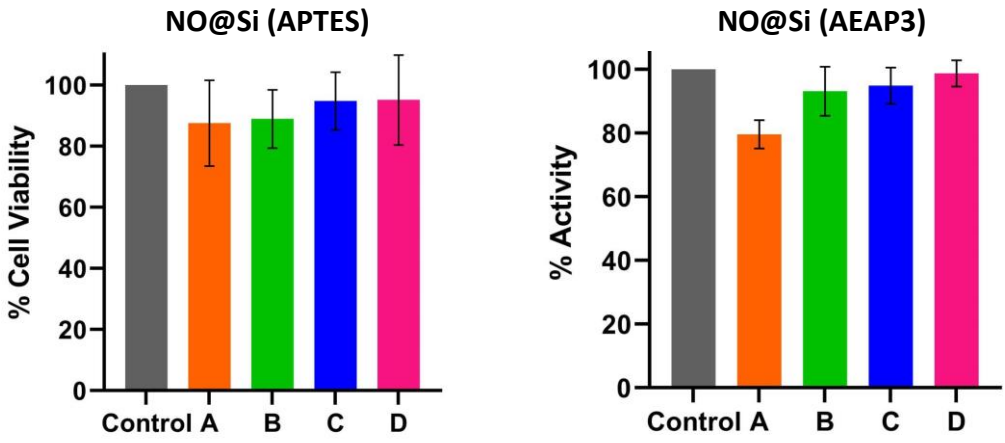



Figure 4-10. Percentage of cell viability (Mean ± SEM) in control group (no treatment) vs. different treated groups. A) 4mg/ml, B) 3mg/ml, C) 2mg/ml, D) 1mg/ml.

# 5- Discussion

The synthesis of NO releasing nanoparticles represents an important step for the development of NO storage or delivery systems in biomedical applications that bridge the gap between small molecule N-diazeniumdiolates and N-diazeniumdiolate-modified macromolecules. Controling over the structure and concentration of TEOS precursors allows for the preparation of NO donor-modified silica nanoparticles of widely varying sizes (d = 70 – 270 nm) and NO release properties such as NO payload of 0.12 – 0.25 mmol·mg-1, half-lives (2 – 24 h), and NO release durations up to 30 hours. Silica nanoparticles prepared by modified Stober method shows an increased NO payload of up to three times greater than previous researchs (2000nm/mg) that were prepared by another methods such as one-pot synthesis. In addition, the diversity of NO release kinetics and scaffold size, and favorable toxicity represent distinct advantages for silica over previously reported nanoparticles such as metal nanoparticles, silver. The ability to manipulate and tune both the NO storage and release characteristics and size of the silica nanoparticles in spherical shape can ease the development of new pharmaceuticals for medical conditions and diseases treatments requiring NO-based therapy. This project suggests that the size of the delivery vehicle is particularly important in determining cellular and tissue uptake and accumulation, with particles having a diameter between 50 and 100 nm as most optimal. The NO releasing silica nanoparticles synthesized herein fit this range. The silica particles also avoid some practical limitations of previously reported nanoconstructs (e.g., dendrimers) in that their synthesis and purification is simple, and their precursors inexpensive. The biodegradable Silica nanoparticles by using AEAP3 is one of the promising result of this project. Since concentration dictates NO's biological action, a scaffold with wide-ranging NO payloads and NO release kinetics may prove useful for a range of applications such as antibacterial effects on wound healing.

# 6- Conclusion

- The modified Stober method will be resulted in silica nanoparticles with lower diameter which will cause in higher surface area that will provide the higher surface for amine grafting and NO storage.

- Silica nanoparticles with diameter about 70 nano meters are able to store higher amount of Nitric oxide (up to 0.24 mmol/gr), resulted in higher release rate and anti-bacterial activity.

- The samples containing more than 0.16 mmol/gr nitric oxide have bactericidal effect on gram negative bacteria (kill the bacteria) and stop the bacteria growth, while for gram positive bacteria the accumulated amount should be more than 0.24 mmol/gr. (due to peptidoglycan cell wall)

- The samples containing less accumulated nitric oxide have bacteriostatic effect (inhibit the bacteria) on gram positive and gram-negative bacteria up to 6-8 hours.

- For the samples with less amount of nitric oxide, adding a second dose is suggested for further study

As a conclusion this project resulted in the higher NO release in the target cells such as gram-negative and gram-positive cells. Moreover, due to antibacterial property of NO, by increasing the concentration of NO at target cells, it is supposed that this scaffold can result in the biofilms harness. The covalent conjugation NONOates can increase the capacity of NPs to store NO. The release NO can inhibit the bacteria growth. Further work can be considered by adding a biomaterial with intrinsic antibacterial property such as silver. Since the release of NO from Silica scaffold can be controlled by thermal treatment and irradiation, silver and its thermal property (surface plasmon resonance) not only can enhance the release of NO, but also its antibacterial property will have an effective impact on bacteria harness.

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