Synthesis, Characterization and Biological Activity Determination of Silver Nanocubes

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Synthesis, Characterization and Biological Activity Determination of Silver Nanocubes

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By

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DECLARATION

I, **Manal Bint Faiz** (Reg # **NUST00000318156),** declare that all work presented in this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been mentioned in the thesis. The work herein was carried out whileI was a Post Graduate student at Atta-ur-Rahman School of Applied Biosciences, NUST, under the supervision of **Dr. Rumeza Hanif**.

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Dedicated to my father, whose tremendous support and cooperation led me to this wonderful accomplishment

Manal Bint Faiz

ABSTRACT

Nanoparticles have been termed as one of the most fascinating findings of science and technology and are finding useful application in almost every field of life. These tiny particles are completely unique from their bulk counter parts as their nanoscale size makes them a great choice for administration in everyday machinery, medical equipment and even in the human body. They have shown great promise in disease diagnosis and prognosis, as drug carriers, and in the treatment of various pathological disorders. Metallic nanoparticles offer an extra advantage of tunable surface plasmon resonance (SPR) which allows them to be targeted by a certain wavelength when inside the body, leaving the tissues undamaged and affecting only the nanoparticles. Silver nanoparticles are among the most widely used metallic nanoparticles in the field of medicine, but the inert antibacterial potentials are by far their greatest hallmark. The antimicrobial and other biological properties seem to be greatly affected by various physicochemical properties of the synthesized silver nanoparticles. Studies have shown that polyol synthesis method is efficient for synthesizing AgNPs of desired shapes and sizes. During the present study we have optimized synthesis of silver nanocubes (AgNCs) using the polyol method. The AgNPs thus synthesized were checked for the desired shape using Scanning Electron Microscopy (SEM). We then investigated antibacterial of the synthesized AgNCs thorough disc diffusion assay, antioxidant activity using FRAP and DPPH assay, hemolytic activity potential, anti-inflammatory activity, and biofilm inhibition potential of these cubical shaped silver nanoparticles through the corresponding biological activity assays. In order to determine the shelf-life of the synthesized AgNCs we also tested the effect of aging on the behavior of these nanoparticles. Our results show that, in general, the cubical AgNPs are less potent in most of these assays as compared to their opposing shapes mentioned in other studies. Our results also indicate that these nanoparticles deteriorate during storage at room temperature in about 6 months as the aged AgNCs did not exhibit any activity at any concentration tested in any assay.

Antibacterial activity of the synthesized AgNCs was determined using seven different bacterial strains viz., *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus pumilus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bordetella bronchiseptica*. The freshly synthesized AgNC samples showed no antibacterial activity against 6 out of seven species of bacteria tested. *Bacillus subtilis* was the only strain that displayed sensitivity to the freshly synthesized AgNCs discs. Interestingly, these bacteria exhibit a better sensitivity at lower concentration of AgNCs rather than at higher concentration. This observation hints at some kind of uptake mechanism that becomes saturated at the higher concentrations. This hypothesis however needs to be further investigated through experimentation. The freshly prepared AgNC samples exhibited antioxidant activity that was directly proportional to their concentration used in the assay. We conclude that the shape of the NP helps to protect the antioxidant activity of the active ingredient i.e., Ag^{+} ion. Our results indicate that these AgNCs are safe for clinical usage as they possess no hemolytic activity and they possess significant anti-inflammatory activity. Our results indicate that the AgNCs possess great potential for use in medicine. Nonetheless there is great need for further experimentation to elucidate in detail exact mechanisms of the observed characteristics and validation of their beneficial properties over AgNPs in other shapes.

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Manal bint Faiz

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CHAPTER 1: INTRODUCTION

1.1 Nanoparticles

Nanoparticles (NPs) are a state of matter with properties unique from their molecular or bulk counter parts. NPs are ultrafine, microscopic material measured between 1 to 100 nm size range [1]. Matter manipulation at the nanoscale was first brought to light by Richard Feynman in 1960 where he highlighted the fact that atoms follow different laws of nature at the quantum level [2]. Nano-sized particles gained importance due to their unique and tunable properties. These unique properties at such miniature scale have been exploited in various fields of science including Physics, Chemistry and Biology. Once the customization of NPs became common practice, it was only a matter of time before these NPs established distinct roles in different fields of science. The manipulation of NPs is now widely termed as Nanotechnology.

1.2 Types of Nanoparticles:

An assortment of different NPs has been developed as per their use and composition. According to a general classification, these NPs fall into two major groups; organic and inorganic NPs [3]. The second classification i.e. the three-category system of organic, inorganic and carbon-based NPs is more recent and accepted worldwide [4].

This three-category classification of nanoparticles is briefly described below.

1. **Organic NPs:** This group includes
    a. Dendrimers are unique polymeric nanostructures with a branched morphology [5].
    b. Micelles are amphiphilic surfactant molecules and instinctively shift in aqueous environment. They primarily form a spherical vesicle with a hollow core [6]. These

types of NPs, for the most part, maintain a hydrophobic center which enables these cores to hold hydrophobic drugs and act as drug delivery vehicles [7].

c. Liposomes [8] are structures much like micelles. They are composed of lipid bilayer that self-assembles in aqueous environment into a spherical form. This spherical vesicle confines liquid in the center which can be exploited in carrying drugs [9]

d. Ferritin, a well-known iron storage protein, has been manipulated and utilized as NPs as a result of its distinctive architecture [10]. A ferritin molecule is composed of an outer and inner surface, both appropriate features for surface modification and drug loading, respectively.

These NPs are biodegradable, non-toxic, and are mostly excellent drug carriers owing to the presence of polymeric configuration and hollow cores in most of the structures.

2. **Carbon-based NPs:** As the name indicates, this category incorporates NPs made solely out of carbon. Nano sized carbon particles include;

   a. Fullerenes, also known as bucky balls, with a diameter of about 7 ampere. They are excellent for carrying DNA within their hollow structure. The most common configuration of fullerenes involves 60 carbon atoms arranged in a spherical structure [11].

   b. Carbon nanotubes are carbon-based tubular structures formed by a graphite sheet rolled up into a cylinder. These tubes are hexagonal arrangements of carbon atoms in a helical manner, providing needle like hollow structures with diameters of around 1 nm and lengths ranging up to 100 nm [12]. Changing the size, thickness,

and hollow core structure of nanotubes has led to alternate versions of carbon nanotubes referred to as nanorods [13], nanowires [14], and nanofibers [15]

3. **Inorganic NPs:** This group of NPs can be further categorized into four nanosystems, namely metallic, bimetallic, magnetic and metal oxide NPs, for ease of understanding [16].
    a. Metallic NPs: Nanosized entities made of pure metal build this subcategory of inorganic NPs. This class, for the most part, consists of Gold (Au) and Silver (Ag) NPs. Such NPs display electronic as well as optical properties that are sought after especially for diagnosis of cancer [17]. These properties along with their chemical inertness [18] and easily modifiable surfaces [19] make pure metallic NPs a very promising tool especially in the biomedical fields.
    b. Bimetallic NPs: They can also be referred to as alloy NPs, include NPs such as Iron Cobalt (Fe-Co) [20], Iron Nickle (Fe-Ni) [21], Copper Nickle (Cu-Ni) [22], Iron Platinum (Fe-Pt) [23] to name a few. These NPs possess particularly attractive features for their use in hyperthermia treatment [24], MRI contrast agents [25], drug delivery [26], and Biosensors [27].
    c. Magnetic NPs: Magnetic NPs generally depict the NPs that comprise of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) and are among the most commonly synthesized and utilized NP [28]. Iron Oxides exhibit remarkable chemical, biological and magnetic properties which make them popular candidates for biomedical applications [29].
    d. Metal Oxide NPs: As the name indicates, these kinds of NPs encompass a large diversity of oxides that metal elements form. Titanium DiOxide ($TiO_{2)}$ [30],

Cerium Oxide (CeO_2) [31], Mesoporous Silica NPs (SiO_2) [32], Zinc Oxide (ZnO) [33], and other metal oxides make up this group of inorganic NPs.

Metallic NPs have been of great interest as they show peculiar physiochemical properties in the nanometer size range. Their size, shape, pore size, surface charge and other characteristics can be modified according to one's interest. NPs synthesized from metal oxides such as Iron Oxide, Zinc Oxide and others are the examples. Metal oxide NPs are highly reactive and are used in the fabrication of their respective metal NPs.

Quantum Dots:

Another class of inorganic NPs are the Quantum Dots which are synthesized from elements in the classical semiconductor groups III-V and II-VI of the periodic table [34]. They predominantly comprise of a semiconductor inorganic core (CdSe) and an organic coated shell (e.g. ZnS) to improve their optical properties. Quantum dots have been observed to produce fluorescence when stimulated by light [35]. They have been successfully utilized in immunoassays [36, 37], DNA hybridization detection [38, 39], for cancer diagnosis and treatment [40], gene therapy [41] etc.

Hybrid NPs:

Dendrimer-encapsulated metal NPs are a form of organic/inorganic hybrid NPs. They are the result of a dendrimer coat with a metal ion core followed by subsequent chemical reduction [42].

1.3 Sources of Nanoparticles:

NPs can be generated from natural resources or they can be generated following a pure synthetic/chemical route. The advancement in the field of nanotechnology has created numerous possibilities for NP synthesis using a diverse collection of materials/mediums/substances.

Metallic or non-metallic NPs can be synthesized both biologically and/or chemically. Various enzymes and/or proteins are suspected to be involved in the bio-reduction and stabilization of metal NPs in different organisms [43]. This 'Green synthesis' or 'Biosynthesis' is being exploited to produce bio-compatible and water-soluble NPs through an eco-friendly course of action [44].

Microorganisms and plants have been used in the bio-synthesis of metal NPs as well, although the exact mechanism is still unclear [45]. Production of NPs using biological sources allows more reproducibility, is non-toxic, and gives high yield.

NPs also exist naturally in our environment. Naturally existing NPs originate incidentally through mechanical/vehicular discharge or can be a result of weather/climate/atmospheric/environmental change. Additionally, naturally occurring biological processes of numerous organism yield a wide range of NPs. Chemically engineered NPs are a result of human involvement and desire to create the most efficient NPs or to study their extraordinary properties and to exploit their novel/unique features [46-51]. Based on their synthetic versatility NPs can be categorized as;

1. **Incidental NPs**

   NPs can be acquired through the environment. Gases from volcanic eruptions, desert dust storms, and even cosmic dust contain a large amount of NPs that reside and interact with the major components of air i.e. nitrogen, oxygen etc. [52].

2. **Engineered NPs**

   The working of engines, automobiles, airplanes and other machines results in combustion of fuel oil and coal etc. All these and other anthropogenic factors lead to the formation of different and mostly toxic NPs released into the environment. Engineered NPs include those produced by automobiles, different machinery,

cigarettes and even the NPs synthesized in laboratories for human use, such as NPs used in cosmetics or in the biomedical field.

3. **Naturally Occurring NPs**

   Naturally occurring NPs may be confused with incidental NPs as they are both produced as a result of naturally occurring phenomena. But naturally occurring NPs are specifically those NPs that are produced by different living organisms from the simplest of micro-organisms to the most complex multicellular life-forms.

1.4 Methods for Nanoparticle synthesis:

The intriguing properties that NPs possess are easily affected by their morphology, size, and basic material. The properties of NPs designed through the synthesis methods influence the application of said NPs. As such, a miscellany of methods has been generated that are used to synthesize the desired type of NP for a specific type of operation. These methods have been broadly classified into two major types, the top-down (destructive) and the bottom-up (constructive) approach [53]. NP synthesis, however, is preferably classified as Chemical, Physical or Biological methods [54].

1.4.1 Physical Methods

The most common physical methods for synthesis of NPs is the laser ablation method and the evaporation-condensation method which is also known as Inert-Gas Condensation (ICG).

The ***Inert Gas Condensation*** (IGC) method is a 'bottom-up' approach. It involves two major steps, evaporation of the source material and subsequent rapid and controlled condensation to form NPs. IGC allows synthesis of ultrafine NPs by controlling different parameters of the reaction such as evaporation temperature, inert gas pressure and others. Silver [55], Gold [56], Manganese [57], Magnesium [58], Copper [59], and a number of other metal NPs have been reported to be

synthesized via Inert Gas Condensation [60]. The major drawbacks of this method are the inaccessibility and high cost of the Inert Gas Chambers.

Another commonly used physical procedure is the ***Laser Ablation*** method. As the name states, this method involves the use of laser beam to break down large sample material into smaller NPs (Top-down approach) in a gaseous or liquid environment. For laser ablation in aqueous solutions a surfactant, generally Sodium Dodecyl Sulfate (SDS), is customarily used to prevent the NPs from forming clusters and help in uniform particle size generation. Gas phase laser ablation procedures make use of closed gas chambers for NP synthesis.

Laser ablation is an efficient means of producing monodispersed NPs as it eliminates the need for complex set-ups, high temperatures and toxic chemicals. This technique is considered time-saving and eco-friendly [61]. Silver NPs with controlled size can be generated using laser ablation method [62].

1.4.2 Chemical Methods

Chemical reduction is the most commonly used chemical synthesis method to produce metallic NPs. This process can be divided into three different phases using different reagents; a reducing agent which reduces the metallic salt; a stabilizing agent and a capping agent to prevent agglomeration to assure correct size distribution, respectively. As is evident in some protocols, a single chemical reagent may act as both reducing and stabilizing agent [63]. This method has indubitably been used countless times in the synthesis of silver NPs with a range of sizes and shapes such as nanospheres, nanowires, nanocubes, dendrites etc. [63-71]. Synthesis of metallic NPs such as Copper [72], Iron [73], Nickel [74], Gold [75], and others via chemical reduction method have been reported as well.

With the creation of colloidal nano-sized dispersions of water-in-oil (or vice versa) with a surfactant as stabilizer [76], the ***microemulsion technique*** offers great uniformity and size controllability of NPs formed. The nanodroplets formed act as nano-reactors in which the chemical reactions take place [77]. It follows a two-phase synthesis method based on interaction of metal precursor and reducing agent present in two immiscible liquids. Reports of Silver [78], Copper [79], Iron [80], Platinum [81], Cadmium, Zinc [82], Palladium [83], Nickel [84], and Gold [85] NPs via microemulsion method have been made. The major disadvantage of this method is the use of highly deleterious organic solvents.

Irradiation method comprises a predefined duration of laser radiation on a solution of metal precursor salt and surfactant. This produces metallic NPs of distinct size and shape. Silver NPs have been generated by γ-irradiation of $AgNO_3$ used as the precursor salt [86] and the size of NPs can be controlled by customizing the duration of radiation pulse applied on the sample [87].

Microwave assisted synthesis is a finer choice for heating than the conventional oil-bath heating method. It offers lower energy usage, reduced chemical waste, more efficient reaction, more precise size control and uniformity [88] [89]. Using this method, the shape of the NPs can be determined as well [90] [91].

UV-initiated photoreduction has also been implemented in NP synthesis. The size of the particles can be controlled by altering the duration of irradiation.

Photoinduced or photocatalytic reduction is considered a clean and cost-effective method for NP synthesis [92]. It is versatile, easy to use and has high spatial resolution. The shape and consequently the optical properties of the NPs can be modulated by the irradiation through light emitting diode [93]. NPs with size as small as 8 nm have been synthesized using this method [94].

NPs have also been successfully synthesized using ***electrochemical synthetic method***. NPs are deposited at the interface of electrolyte solution containing precursor salt and a metal electrode substrate [95]. The size of NPs can be controlled by changing the electrolysis parameters while the overall size distribution is controlled by altering electrolyte solution composition [96] [97] .

Sonoelectrochemical technique combines sonic and electric pulses in the synthesis of NPs where size and shape formation are controlled by the electrolyte composition and ultrasound specifications. NPs produced through this method are predominately metallic in nature [98].

Several ***polysaccharides and polymers*** have also been employed in the synthesis of different types of NPs. In an environmentally friendly method, water is used as the solvent while a range of polysaccharides function as reducing and stabilizing agents [99]. For example, polymers with the capability of ion-exchange have been used in the synthesis of (silver) NPs.

Tollens' reagent test is a common test used to distinguish aldehydes and ketones. The main reagent in this procedure is silver diamine ($[Ag(NH_3)_2]^+$). This reagent has been exploited in the synthesis of silver NPs in the following method that uses polysaccharides/sugars as reducing agents for silver in the presence of ammonia [100]. In a modified green synthesis method, tollens reagent is used along with phytochemicals which act as the reducing agents [101]

1.4.3 Biological Methods

Although there are a range of physical and chemical methods to synthesize NPs of different shapes and sizes, some scientists argue that these methods make use of environmentally harmful and cost-ineffective chemicals. Thus, biological methods to synthesize NPs have been explored. In these methods living organisms are brought into play to synthesize NPs. Many of these organisms include bacteria, fungi, algae and a miscellany of plants.

The biological synthesis methods are considered more quick, cost-efficient, don't require high temperatures, and are less harmful than traditional physical and chemical methods [102]. These approaches are more similar to chemical synthesis methods in that they are bottom-up mechanisms that follow the oxidation/reduction of precursor salts. Various enzymes from microorganisms and plant phytochemicals act as antioxidant or reducing agents and encourage NP synthesis [103]. Exact mechanism for the biological synthesis of NPs is not entirely known at the moment but there is substantial in silico data that suggests the role of biomolecules as capping and/or reducing agents.

In the case of microorganisms, synthesis can occur extracellularly or intracellularly. During the extracellular process, cultures are grown under optimum conditions, centrifuged, and the biomass is removed. The supernatant of these cultures is then used along with precursor metal salt to synthesize NPs. The intracellular method is relatively similar. The major difference is that instead of removing the biomass, it is collected after centrifugation, washed, and then used to generate NPs by adding the precursor salt. For both these methods, the change in color of the sample is used as an indicator for NPs synthesis. In the intracellular synthesis method, an additional step to break the cell walls of the microorganisms to release and collect the NPs synthesized inside the cells needs to be carried out.

For synthesis via plants, plant extract (from different plant parts such as roots, bark, leaves) are used along with the precursor metal salt and water depending on the type of extract. Incubation of this reaction mixture leads to the reduction of metal salt and the change in color indicates the synthesis of the NPs [45]. Listed below are some microorganisms and plants reported to have been involved in the synthesis of various silver NPs.

The most diversely used microorganisms, ***Bacteria,*** as in other fields of life science, are largely used in the synthesis of numerous NPs as well. *Pseudomonas sp.*, for example, have been documented to synthesize AgNPs with an average size of 50 nm.

Table 1: NPs with varying sizes synthesized by bacterial and actinomycetes species.

Bacterial species	**Nanoparticles**	**Size**	**Reference**
Pseudomonas sp.	Ag	~50 nm	[104]
Bacillus licheniformis	Ag	40 nm	[105]
Bacillus spp.	Ag	77-92 nm	[106]
Ochrobactrum anhtropi	Ag	38-85 nm	[107]
Pantoea ananatis	Ag	8.06-91.32 nm	[108]
Bacillus brevis NCIM 2533	Ag	41–68 nm	[109]
Bacillus mojavensis BTCB15	Ag	105 nm	[110]
Actinobacter	Ag	13 nm	[111]
Sinomonas mesophila MPKL 26	Ag	4–50 nm	[112]
Bacillus endophyticus SCU-L	Ag	5.1 nm	[113]
Bacillus licheniformis Dahb1	Ag	18.69–63.42 nm	[114]

Bacillus methylotrophicus DC3	Ag	10–30 nm	[115]
Stenotrophomonas GSG2	Ag and Au	Au (10–50); Ag (40–60)	[116]
Bacillus subtilis	Au	20–25 nm	[117]
Shewanella loihica	Au	2–15 nm	[118]
Kocuria flava	Cu	5–30 nm	[119]
Shewanella loihica	Cu	10–16 nm	[120]
Shewanella loihica	Pt	1–10 nm	[118]
Shewanella loihica	Pd	1–12 nm	[118]
Staphylococcus aureus	ZnO	10–50 nm	[121]
Lactobacillus sp.	TiO_2	8–35 nm	[122]
Actinomycetes			
Streptomyces spp.	Ag	11-63 nm	[123]
Nocardiopsis sp. MBRC-1	Ag	45 nm	[124]
Streptacidiphilus durhamensis	Ag	8-48 nm	[125]
Streptomyces rochei MHM13	Ag	22-85 nm	[126]
Streptomyces sp.	Ag	15-25 nm	[127]

Gordonia amicalis HS-11	Au-Ag	5–25 nm	[128]
Rhodococcus sp.	Au	5–15 nm	[129]
Nocardiopsis sp. MBRC-48	Au	11.57 nm	[130]
Streptomyces capillispiralis Ca-1	Cu	3.6–59 nm	[131]
Streptomyces sp.	CuO	78–80 nm	[132]
Streptomyces sp.	ZnO	20–50	[133]

Fungal strains have also been reported to have a significant role in the shape of NPs synthesized [134]. They excrete proteins and enzymes in large quantities that help in reduction of metal salt and provide a process that can be easily scaled-up [135]. These and other qualities of fungal sp. have convinced scientists in using them for the production of NPs. Listed below are some fungal and yeast strains that have been used in the successful synthesis of NPs.

Table 2: Different types of NPs synthesized by fungal and yeast species.

Fungal Species	**Nanoparticles**	**Size**	**Reference**
Rhizopus stolonifer	Ag	2.86 nm	[136]
Candida glabrata	Ag	2–15 nm	[137]
Trametes trogii	Ag	5–65 nm	[138]
Trichoderma longibrachiatum	Ag	10 nm	[139]

Fusarium oxysporum	Ag	21.3 to 37.3 nm	[140]
Aspergillus terreus	Ag	16-57 nm	[141]
Cladosporium cladosporioides	Au	60 nm	[142]
Rhizopus oryzae	Au	16–43 nm	[143]
Saccharomyces cerevisiae	Au	-	[144]
Penicillium chrysogenum	Pt	5–40 nm	[145]
Aspergillus niger	ZnO	53–69 nm	[146]
Pichia kudriavzevii	ZnO	10–61 nm	[147]
Alternaria alternata	Fe_2O_3	75–650 nm	[148]
Saccharomyces cerevisiae	Pd	32 nm	[149]

The eukaryotic, aquatic, photoautotrophic organisms, ***algae***, have been known to be capable of bioremediation by accumulating heavy metals from the surrounding environment. However, not many accounts of NP synthesis using algae have been reported. Red algae *Portieria hornemannii* [150], marine macroalgae *Padina* sp. [151], and *Gelidium amansii* [152] are a select few among a number of other algae that have successfully been used in the synthesis of AgNPs.

Another fascinating method reported for the synthesis of NPs is through the use of ***viruses***. These nanoscale sized organisms are sought after because of their small yet controlled sizes and

morphologies. The unique chemistry of the viral capsid allows for its chemical modification and exploitation in generating NPs [153].

The first report of NPs synthesized by ***plant*** extract was made in 2003 through which silver NPs synthesis was documented using alfalfa sprouts [154]. Since then a countless number of plant species have been investigated and explored for the synthesis of NPs. Plant extracts have been known to contain certain phytochemicals that act as reducing and stabilizing agents which are key elements in the synthesis of NPs [155]. Till present, a great deal of plants has been used in the synthesis of all sorts of NPs, a few of which are mentioned below.

Table 3: List representing sizes and different NPs synthesized by plant species.

Plant Species	**Nanoparticle**	**Size**	**Reference**
Annona reticulata	Ag	7–8	[156]
Camellia sinensis	Ag	2–4	[157]
Mulberry fruit *(Morus alba L.)*	Ag	80–150	[158]
Panax ginseng	Ag	5–15	[159]
Nigella arvensis	Au	3–37	[160]
Rhazya stricta Decne	Au	40	[161]
Mulberry fruit *(Morus alba L.)*	Cu	50-200	[158]
(Syzygium aromaticum) clove	Cu	~ 15–20	[162]
Moringa oleifera	Fe	2.6–6.2	[163]

Avicennia marina	FeO	10–25	[164]
Aloe socotrina	ZnO	15–50	[165]

1.5 Biomedical Applications of Nanoparticles:

The specific properties of an element are contributed by the subatomic particle, the electrons. In metals especially, these electrons are greatly delocalized [166], having an average free path of about 10 to 100 nm. Therefore, in such elements, changes in size and shape are cause for substantial deviations in properties from their bulkier analogues, and new size-dependent properties emerge.

Owing to the extraordinary chemical and physical properties that NPs possess, there is no doubt that they would be extensively and ever so thoroughly exploited in the field of biomedicine. Their increased surface-to-volume ratio, easily modifiable surfaces, magnetic and optical properties make them superior to their larger counterparts especially in the diagnosis and therapy of diseases. In vitro trials of NPs in Drug Delivery [167], Bioimaging [168, 169], Biosensors [170], Photothermal Ablation [171], Hyperthermia [172], as Labelling and Tracking Agents [173], Gene Therapy [174] and others have been no less than positive.

One of the most intriguing and significant feature these NPs put forward is their adjustable and chemically modifiable surface. This remarkable trait to biofunctionalize NPs makes them biocompatible, easy for targeting and tracking inside the body, and less harmful for use [175].

1.6 Silver NanoCubes:

Silver nanoparticles (AgNPs) can be synthesized in specific shapes, each with their own unique properties such as nanospheres [176], nanorods, nanowires [177], nanoprisms [178], nanoshells [179], and nanocubes [180]. Depending on the size and shape of the AgNPs, their Surface Plasmon Resonance (SPR) and hence other optical properties, such as refraction, absorption, reflection and scattering of light, are altered [181]. The properties of AgNPs greatly depend on their shape and size. As the production of silver nanostructures exponentially grow it constitutes rapid growth in research in the field of nanomaterials.

Studies to control the shape of AgNPs to produce cubic structures have resulted in the formation of Silver Nanocubes (AgNCs) [182]. AgNCs have been pursued for their Surface Plasmons properties [183] and their optical properties [184].

AgNCs are generally prepared at high temperatures using a bottom-up chemical reduction method commonly referred to as the polyol synthesis [185]. Alterations in different parameters of the reaction, such as the chemicals [186], temperature [187], reaction time etc. [188], result in nanocubes of different sizes and also determine whether they will have sharp edges or curved corners. Silver mirror reaction is another approach that has successfully been used to synthesize AgNCs [189].

1.6.1 Applications (of silver NPs) in Biomedical Sciences:

Since the evolution of nanotechnology, AgNPs have become one of the most extensively researched and explored nanomaterials synthesized in view of the fact that these metal nanostructures provide unique, challenging, and promising properties desirable for various biomedical applications.

1.6.1.1 Antibacterial Agents:

Perhaps the most commonly conducted biomedical application of AgNPs are the ***antibiotic assays*** conducted due to their well-known intrinsic antimicrobial effect [190]. As a consequence of their antibacterial quality, they have been implemented in tissue scaffolds, protective clothing and wound dressing [191]. In context to this property, AgNPs incorporated in nanofibers have shown positive results against both gram positive and gram negative bacteria [192]. Studies indicate that storage and shelf life of AgNPs can be altered by modifying their surfaces. These studies have also shown that the AgNPs "age" over time during storage thus effecting their toxicity [193].

Various factors affect the antibacterial effect possessed by the AgNPs. For example, smaller sized NPs display greater antibacterial toxicity [194]. Similarly, shape of the NPs [195], concentration and the method selected to test antibacterial effect [196] and surface modifications [197] affect the antibacterial property of the NPs. Method of synthesis also affects the toxicity of AgNPs. Chemically synthesized AgNPs have been reported to show greater cytotoxic effect in comparison with green synthesized AgNPs [198].

It is worth mentioning that AgNPs when used in human cells cause cytotoxicity, genotoxicity [199], and inflammatory responses [200] in a cell-type-dependent manner. AgNPs have also been seen to have a positive role in wound healing in animal models [201].

There is an ongoing debate between scientists where some state that the AgNPs themselves show antibacterial effects while others believe the NPs release silver ions that go on to kill the bacteria. Despite the uncertainty of the exact process that takes place, studies have shown that silver ions are more potent killers of bacteria than the whole AgNP, and support the possible release of silver ions from AgNPs [202].

The intrinsic antibacterial property that AgNPs possess has widely and extensively been applied in various medical specializations/sectors including catheter modifications [203, 204] where AgNPs are combined with the catheter and promise a maintained sterilization effect, in dentistry [205], wound healing applications [206], infected bone repair [207] and more.

1.6.1.2 Drug Delivery Systems:

Understanding the Pharmacokinetics and Pharmacodynamics of drugs has always been a very crucial part of medicine. Following the target specific and selective action of drugs in the body and the advances in the field of nanotechnology, NPs have since been extensively studied regarding their novel design and development for their role as drug carriers as metallic NPs [208], polysaccharide-based NPs [209], Solid Lipid NPs [210], Protein-based NPs [211], including NPs synthesized via green synthesis [212].

AgNPs have been successfully used as drug carriers for a multitude of therapeutic medicines a few of which include anti-inflammatory [213, 214], anti-oxidant [215, 216], antimicrobial [217, 218] and anticancer drugs [219, 220].

In the case of AgNCs, they have been additionally customized with Gold metal to produce a Gold-Silver (Au-Ag) hybrid cubic nanoparticle, which has effectively been used in drug delivery process [221].

1.6.1.3 Role in Cancer Treatment:

AgNPs have already demonstrated to be innately antibacterial [222], antiviral [223], antifungal [224], anti-angiogenic [225], anti-inflammatory [226], and even antiplatelet [227].

In accordance with these results, AgNPs were subjected to intensive investigation regarding their role in cancer and have proven to possess intrinsic anticancer activities [228, 229]. They have been tested against different human cancerous cell lines such as endothelial cells [230], IMR-90 lung fibroblasts, U251 glioblastoma cells [231], and MCF7, MDA-MB-231, and MCF 10A breast cancer cells [232].

Their role as carriers of anticancer drugs has been broadly explored and adds to their anticancer treatment [233]. The combined effect of anti-cancerous AgNPs with a known anti-cancer drug has proven to greatly enhance the cytotoxicity of cancer cells, making them more sensitive to the drug and induce apoptosis [234]. Targeted drug therapy using nanocarriers gives access to control the fate of the drug in the body, concomitantly protecting healthy tissues and cells [235].

Although it is preferred that the metal NPs being used for *in vivo* treatment be inert, such NPs may cause bioaccumulation and toxicity. On the contrary, NPs possess the ability to efficiently absorb light energy and convert it to heat energy, which has proven to be a very promising and desirable feature in the treatment of cancer and other such diseases. As a result, metal NPs show promise in hyperthermic tumor therapy (photothermal therapy), where stimulation of NPs via photons produces thermal energy [236].

AgNCs have also demonstrated their functionality in diagnosis and treatment of cancer. They have been tested as potential detectors of lung cancer biomarkers [237], and as biosensors for oral cancer [238].

The biggest contribution of AgNCs in cancer treatment so far, has been their use as templates for the synthesis of Gold Nanocages (AuNCs) [239]. These AuNCs are specifically designed to achieve maximum absorbance of near-infrared wavelength of light for photothermal therapy [240].

These AuNCs have also been used in combination as drug carries and photothermal agents to achieve maximum cancer cell destruction [241].

However, there is still a great need to further investigate AgNCs and their potential role in cancer diagnosis and treatment.

1.6.1.4 Biosensors and Bioimaging:

It is important in the field of medicine to be able to view biomolecules and to understand their physiology and interaction mechanisms [242]. Alongside the general imaging techniques such as X-Rays, Computed Tomography (CT) scans, and Magnetic Resonance Imaging (MRI) and ultrasound, nanoparticle-based contrast agents are being used for bioimaging. On top of that, due to its Surface Plasmon Resonance (SPR), Ag demonstrates unique optical and electronic properties when excited by an external light source [243]. Consequently, the noble metals, Ag and Au, have established a special role as biosensors and in bioimaging. AgNPs have been used to image biomolecules in renal cell carcinoma [244], in the detection of IgG immunoglobulin [245], protein imaging in human bone marrow neuroblastoma cells [246] and more.

Perhaps the most prominent role AgNPs display is in the field of Biosensor and Bioimaging. Along with Ag, AuNPs have also been greatly investigated in this area. This is because metallic NPs offer tunable and unique plasmonic properties that are highly suitable in this regard. AgNPs combined with a biomolecular probe have been very reliable in the sensitivity and stability of target molecule detection [247, 248].

In accordance with the applications of AgNPs in Biosensing and imaging, AgNCs have also played their role in this field and have shown positive outcomes. Their contribution as biosensors for the detection of biomarkers in various cancers has been worthwhile in cancer diagnosis [238]. As

complete optical transparency for AgNPs in tissues is achieved by changing their shape from spheres to hollow cubes, AgNCs have provided great opportunities to be used in optical switching and bioimaging [249].

Nanotechnology is also a rapidly rising research field with great capabilities and promise in various other domains of science such as Physics, Chemistry and Biology. Prodigious amounts of investigation and experimentation are being carried out on the methods of synthesis and practicality of the synthesized NPs. Undeniably, NPs have proven themselves competent and applicable especially in the field of health sciences. Regardless, there is still much need for further inquiry of the effects of shape of NPs. Although scientists are already well aware of all the beneficial attributes of AgNPs, the shape-specific contributions have not yet been clearly delineated. This study focuses on shape specific AgNCs and attempts to demonstrate and elucidate their potential bioactivities by employing them in conventional bioassays.

CHAPTER 2: MATERIALS AND METHODS

2.1 Silver Nanocube Synthesis:

AgNCs were synthesized using two different protocols. The AgNCs thus obtained were characterized using SEM facilities available at LUMS, Lahore and later used in different biological assays. These assays were carried out to determine biological activities and the shelf life of the synthesized AgNCs. Protocols for the synthesis and assessment of the biological activities are narrated in the following sections.

Chemicals: The chemicals used during the present study were either AR or Molecular Biology grade and were purchased from Sigma-Aldrich, Merck, Fluka or corresponding renowned manufacturers.

2.1.1 Synthesis of Silver Nanocubes – Protocol 1 [250]:

To synthesize AgNCs using this protocol an oil bath was set at 150-155℃ on a hot plate equipped with magnetic stirring. Ethylene Glycol (EG, 6 ml) was added to a 24 ml vial along with a clean, egg-shaped stir bar. The vessel containing EG was placed in the oil bath for 1 hour and 30 mins. $AgNO_3$, PVP and Na_2S solutions were prepared as described (Appendix). When the temperature of the solution reached 150-155℃, 100 µl of ~3 mM Na_2S was added. After waiting for 8-9 mins, 1.5 ml PVP was added in two 0.75 ml aliquots. Immediately thereafter, 500 µl (282mM) $AgNO_3$ was added. The reaction assembly was covered with aluminum foil and left for 20 mins. To stop the reaction, the magnetic hotplate was turned off and the vial was immediately placed in a cool (25℃) water bath to stop the reaction.

2.1.2 Isolation of Nanocubes:

The contents of each vial were transferred to a 15 ml tube. This was followed by rinsing with acetone. The acetone washings were also added to the tube. The tube was centrifuged at 2000 g for 30 mins. The supernatant was discarded, the NP pellet was suspended in 2 ml sterile distilled water and sonicated for 10 mins. The NP suspension was centrifuged again at 9000 g for 20 mins. After repeating these steps for at least 4-5 times, the pellet obtained was finally resuspended in 2 ml sterile distilled water. The tube containing NPs was wrapped in aluminum foil and stored at RT for a definite (7 months) period of time.

2.1.3 Synthesis of AgNCs – Protocol 2 [251]:

Another method mentioned by Bottomley, Prezgot and Ianoul (2012), was used to synthesize AgNCs. A Round Bottom Flask (RBF) carrying 35 ml EG and a clean stir bar was placed in an oil bath set at 150℃ for 1 hr with continuous stirring. PVP solution (5 ml) was added to the RBF after heating for 1 hr. Five minutes later, 400 µl of 3 mM Na_2S in EG was added. After another 5 minutes, 2.5 ml of 282 mM $AgNO_3$ solution in EG was added in a controlled manner. The solution was heated for another 15 minutes. The entire procedure was carried out in the absence of light. Small aliquots of the sample were taken out at regular intervals and diluted with ethanol for UV-visible spectroscopy. The reaction was stopped by placing the RBF in cold (25℃) water bath.

2.1.4 Isolation of Nanocubes:

The prepared solution was diluted with ethanol (1:1 volume) and centrifuged at 6000 rpm for 15 mins. The supernatant was discarded, and the pellet was resuspended via sonication in ethanol. This procedure was repeated 5 times. Two final washings of the sample were done the next day

with sterile distilled water and the sample was stored as 4℃ in 4 ml sterile distilled water for a pre-defined time period.

2.1.5 UV-Visible Spectroscopy:

Various dilutions of the prepared sample were prepared and their optical density (OD) was measured at 200-800 nm (UV) range.

2.1.6 Characterization of the nanocubes:

A 10% dilution of both samples was prepared by adding 100 µl of NP solution and 900 µl of deionized water in an Eppendorf tube. This solution was mixed using micropipette. 100 µl of this 10% solution was then extracted and mixed with 900 µl of deionized water in another Eppendorf tube to make a 1% dilution. The prepared samples were sent to LUMS for SEM (Scanning Electron Microscopy with EDX and E-beam Lithograph FEI Nova 450 NanoSEM) characterization.

2.2 Antibacterial Assay:

Antibacterial assay was performed using the Kirby-Bauer disc diffusion method [252]. Seven different species of bacteria were used, namely *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Bacillus pumilus* (ATCC 14884), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 10536), and *Bordetella bronchiseptica* (ATCC 4617). Four different antibiotic drugs, Ampicillin (A), Ceftriaxone (B), Cefotaxime (C), and Meropenem (D), were used as standards or positive controls. Discs were prepared as follows.

A 10% dilution of AgNP sample was prepared and 10 µl of this dilution was loaded per filter paper disc. In parallel, another set of discs carrying 20 µl of the 10% diluted AgNPs sample was also

prepared. Similarly, discs for all 4 antibiotics were prepared by loading 20 µl, 10 µl, or 5 µl of 10 mg/ml of drug solution per disc. For the combined NPs + Antibiotic discs; against 20 µl positive control discs, 10 µl of antibiotics and 10 µl of the NPs solution were loaded per disc. Blank discs were prepared by loading the discs with sterile water. The discs were air dried and stored at RT.

Spread plate technique was used to culture bacterial lawn in the petri plates. The Petri dishes were sterilized in hot air oven at 110 ℃. 15-20 ml Nutrient Agar was poured in each plate and left to solidify. After solidification of agar, ~200-300 µl of overnight bacterial cultures were spread on the solid agar using a sterilized glass spreader. Standard microbiology techniques were used to avoid contamination. The bacterial cultures were allowed to soak into the agar for about 10-15 minutes after which the discs were placed on the agar surface using forceps according to their pre-determined locations. These plates were placed upside down and left overnight in an incubator at 37℃. Growth and probable subsequent zones of inhibition were examined the next day.

This antibacterial assay was performed with 4 dilutions, 10%, 20%, 30% and 40% of the aged sample and 3 dilutions, 1%, 10% and 30%, of the freshly prepared sample. Our main goal was to determine whether these AgNC samples showed any antibacterial activity at any concentration and whether the aging affects AgNCs antibacterial activity, if any.

2.3 Antioxidant Assays:

2.3.1 FRAP Assay:

FRAP (Ferric Reducing Antioxidant Power) assay was performed to determine the antioxidant properties of the synthesized AgNCs. This assay follows reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by the antioxidants present in the samples. If the sample contains antioxidants, the mixture color changes to blue, or otherwise remains green.

The assay was conducted in properly labeled Eppendorf tubes. The reaction mixtures were prepared as per the scheme shown in the following Table.

Table 4: Chemicals and their amounts used to carry out FRAP Antioxidant assay.

Ingredients	**Positive Control**	**Test**	**Negative Control**
Ascorbic acid (1%)	100 µl	-	-
NP dilution or original	-	100 µl	-
Water	-	-	100 µl
0.2 M Phosphate Buffer pH 6.6	250 µl	250 µl	250 µl
Potassium ferricyanide (1%)	250 µl	250 µl	250 µl
Total	600 µl	600 µl	600 µl

During the assay, 4 (v/v) dilutions of NCs sample (1%, 10%, 20% and 40%) were tested. Ascorbic acid (1%) was used as the positive control, while water was used as the negative control.

The reaction tubes were incubated at 50°C for 20 mins. After the incubation, 250 µl of 10% (w/v) TCA was added to each tube. The tubes were centrifuged at 1350 RPM for 20 mins. The supernatant then obtained was collected from each tube and processed as follows for Absorbance or Optical Density (OD) measurement.

Table 5: Chemicals and their amounts used to carry out FRAP Antioxidant assay.

Ingredients	**Volume**
Supernatant	250 µl
Distilled water	250 µl
$FeCl_3$ (0.1% w/v)	50 µl

OD of the reaction mixture was determined at 700 nm using an ELISA plate reader.

2.3.2 DPPH Assay:

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was performed to determine the antioxidant activity of the samples. This method was adopted from Shimada et al., [253] and optimized.

DPPH solution (90 µl of 0.3 mM) and the sample solution (10 µl) were mixed and incubated for 30 min in the dark at room temperature. The absorbance of this solution was measured at 517 nm using an LT-4500 96-well microplate reader, Labtech UK. Absorbance of the blank and the standard were also measured in the same way. These tests were performed in triplicates. The percentage of total inhibition of DPPH radicals was calculated using the equation given below.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of the blank} - \text{Absorbance of the sample}}{\text{Absorbance of the blank}} \times 100$$

Antioxidant activity of a suitable dilution of each active sample was determined and IC_{50} values were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, (USA).

2.4 Hemolytic Activity:

The purpose of performing the hemolytic activity was to determine whether AgNCs possess the ability to destroy erythrocytes. Previous studies [254] have shown that AgNPs are hemolytic in nature, but their hemolytic effect is generally lower as compared to their antibacterial capabilities. As is the case with their antimicrobial activity, the hemolytic property of AgNPs is affected by different parameters/physiologic characteristics of the AgNPs especially their size [255].

The synthesized AgNCs were assessed for their hemolytic activity using the methods described elsewhere [256, 257]. Freshly obtained heparinized bovine blood 93 ml) was collected from the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. The blood was centrifuged for 5 min at 1000 x g. The plasma was discarded, and the obtained cells were washed three times with 5 ml chilled (4°C) sterile isotonic Phosphate-Buffer Saline (PBS) pH 7.4 every time. The erythrocytes were maintained at 10^8 cells per ml for each assay. Each AgNC suspension (100 µl original or a dilution) was mixed with human erythrose 10^8 cells/ml and tested separately. Samples were incubated for 35 min at 37°C and agitated after every 10 min. Immediately after incubation, the samples were placed on ice for 5 min and then centrifuged for 5 min at 1000 x g. The supernatant (100 µl) was taken from each tube and diluted 10 times with chilled (4°C) PBS. Triton X-100 (0.1% v/v) was taken as the positive control and PBS was taken as the negative control. Both the controls were passed through the same process.

The absorbance was measured at 576 nm. The percentage of RBCs lysis was calculated for each sample using the following formula.

$$\text{Percentage hemolysis} = \frac{\text{Absorbance of the sample} - \text{Absorbance of the blank}}{\text{Absorbance of the positive control}} \times 100$$

2.5 Anti-inflammatory Activity:

AgNPs and NCs are generally known for their destructive and cytotoxic properties. By performing the anti-inflammatory assay on AgNCs, we aimed to discover the reduction of inflammation, if any, caused by our AgNCs. A study had shown the nanocrystalline silver to be anti-inflammatory in nature as it helps in improving the overall healing process [258]. Various green synthesized AgNPs have been tested for their anti-inflammatory activity and results have been very promising [259, 260]. However, the anti-inflammatory effect of AgNPs has not been investigated thoroughly as opposed to their inflammatory response.

For the evaluation of anti-inflammatory activity of AgNCs, 0.2% (w/v) stock solution of Bovine Serum Albumin (BSA) was prepared in tris-buffer saline and the pH was adjusted from pH 8.53 to pH 6.74 using glacial acetic acid. For each assay two replicates of 500 µl of one of these BSA stock solutions were pipetted and 5.0 µl of the test material, AgNC suspension, in methanol was added to the BSA solution. The control consisted of 500 µl BSA with 5 µl methanol. The samples were heated at 72℃ for 5 min in 2.0 ml Eppendorf tubes in metal racks, followed by cooling for 20 min under laboratory conditions. The absorbance was read using a Spectrofluorimetric wavelength parameter of Ex 480/Em 520 in 1.0 ml glass cuvettes or at 660 nm in a Spectrophotometer. The percentage inhibition of precipitation (stabilization of the protein) was determined on a percentage basis relative to the controls as in the following equation.

$$\text{Precipitation inhibition (\%)} = \frac{\text{Absorbance of the control} - \text{Absorbance of the sample}}{\text{Absorbance of control}} \times 100$$

According to the assay mentioned above, samples/compounds that inhibited denaturation with a percentage greater than 20% over a range of concentrations were regarded as those having anti-inflammatory qualities [261].

2.6 Biofilm Inhibition Assay using Microtiter-plate method:

Some microorganisms, such as bacteria, fungi and protists, tend to form a layer of growth known as biofilm. These complex communities are resistant to general antibiotics and are the cause of more chronic pathogenesis [262]. AgNPs have already proven to be antibacterial in nature and as anticipated they are a great alternative against biofilms [263]. As for the effect of shape on the antibiofilm property of AgNPs, a recent study suggests that AgNCs show a more pronounced effect when compared to their spherical counterparts [264].

The biofilm formation was accomplished using method described elsewhere [265, 266]. In a sterile 96-well flat-bottomed plastic tissue culture plate wells were filled with 100 µl of nutrient broth (Oxoid, UK), 100 µl testing sample and 20 µl of bacterial suspension. The positive control wells contained 100 µl of nutrient broth and 20 µl of bacterial suspension whereas the negative control wells contained nutrient broth only. The plates were covered and incubated aerobically for 24 hours at 37℃. The content of each well was washed three times with 220 µl of sterile phosphate buffer. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 220 µl of 99% methanol per well, and after 15 min plates were emptied and left to dry. The plates were then stained for 5 min with 220 ml of 50% crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water.

After the plates were air dried, the dye bound to the adherent cells was resolubilized with 220 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 630 nm using microplate reader (BioTek, USA) according to the methods described elsewhere [267]. All the tests were carried out in triplicate against the selected bacterial strains and the results were averaged. The bacterial growth inhibition (INH%) was calculated as follows;

$$\text{INH \%} = \frac{100 - OD_{630}\ \text{sample}}{OD_{630}\ \text{control}} \times 100$$

CHAPTER 3: RESULTS AND DISCUSSION

Synthesis of metal NPs and their applications have emerged as one of the most active areas of scientific investigation. The growing body of data suggests that these particles have unique characteristics and have a wide range of applications particularly in the area of life sciences. As the data accumulates, the focus is shifting towards synthesis of metal NPs having a pre-determined shape and use of these uniquely shaped particles in biological systems as an attempt to improve the efficacy and usefulness of these micro sized genies. During the present study AgNCs were synthesized, characterized through electron microscopy and checked for a variety of applications through various assays to assess their potential biological activities. Results of all the assays were recorded to assess usefulness of these specifically shaped NPs. In addition, a comparison between aged AgNCs and freshly synthesized AgNCs was done to determine whether AgNCs lose their biological activities after storage for a specific period of storage.

3.1 Synthesis of Nanoparticles:

AgNCs were synthesized using a chemical reduction process known as the polyol synthesis method. Different shapes of AgNPs can be achieved using variations of the polyol method (Fig. 3). Our aim was to achieve cubic shape so as to investigate and evaluate their effect, if any, in various bioassays. Realistically, producing specific silver nanostructures in a laboratory is a challenging task [268]. We used two slightly altered variations of the polyol process [250, 251]. The optical density (OD) of the prepared solution was measured at 200-800 nm (UV) range using a UV-Visible Spectrophotometer.

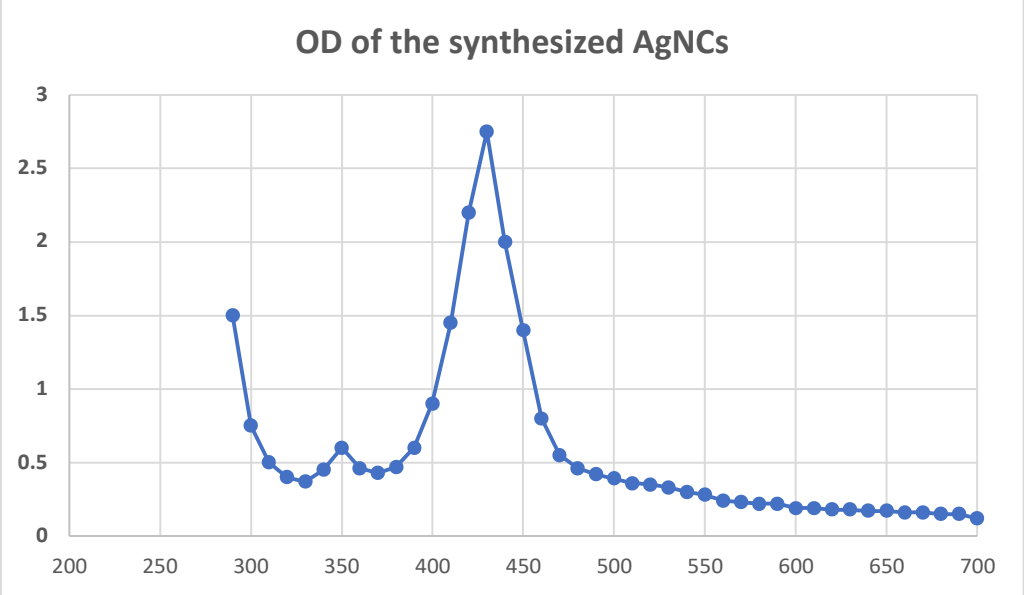

Figure 1: Optical Density of feshly prepared AgNCs sample using UV-Visible Spectrophotometer.

S.E.M. results were acquired using the facility available at LUMS University, Lahore. The samples were characterized immediately after synthesis (Figures 2-5). Cubic nanostructures were noticeably visible in the first batch synthesized, clumps and clusters appearing in the second batch probably indicate a lack of sonication before SEM imaging. We conclude that while both the methods can produce nano cubes, the second method [251] is more efficient in producing bulk of these specifically shaped AgNPs. For aging, a part of the synthesized nano cubes were stored at room temperature for about 7 months. Later, results of the biological assays of the fresh and the aged samples were compared to determine shelf life and effect of storage on these NPs.

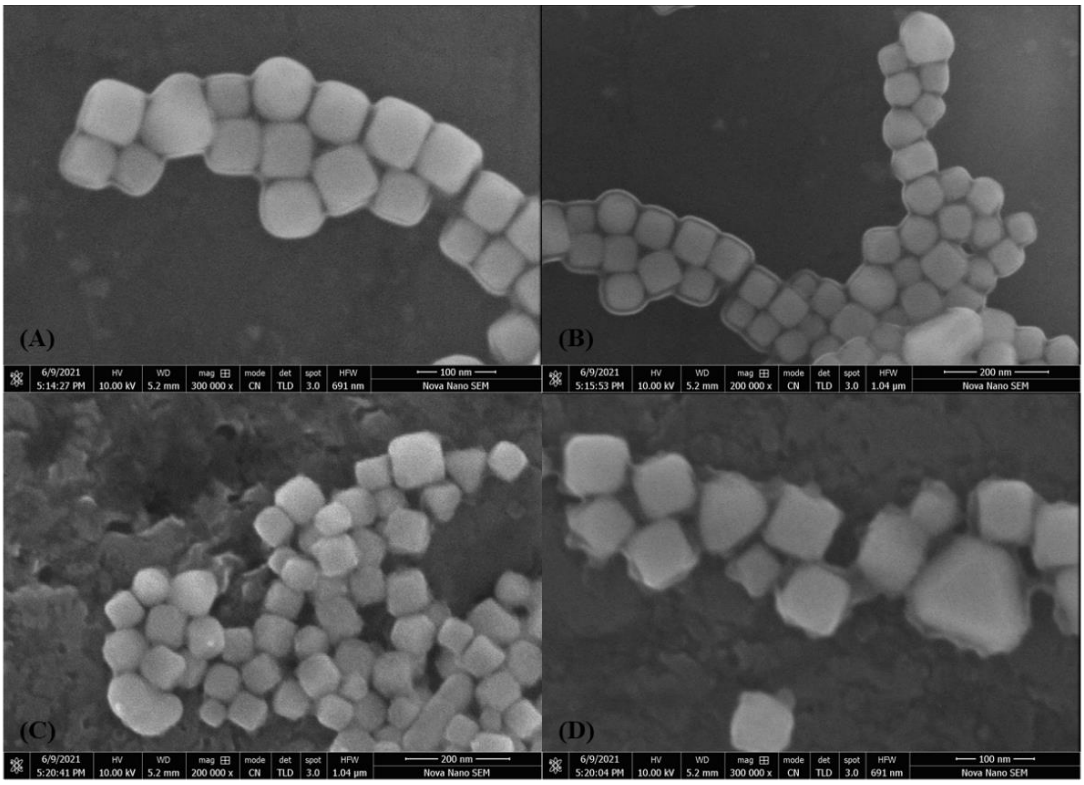

Figure 2 (A, B, C, D): S.E.M results of Silver NanoCubes synthesized using polyol synthesis showing silver nanocubes with curved edges.

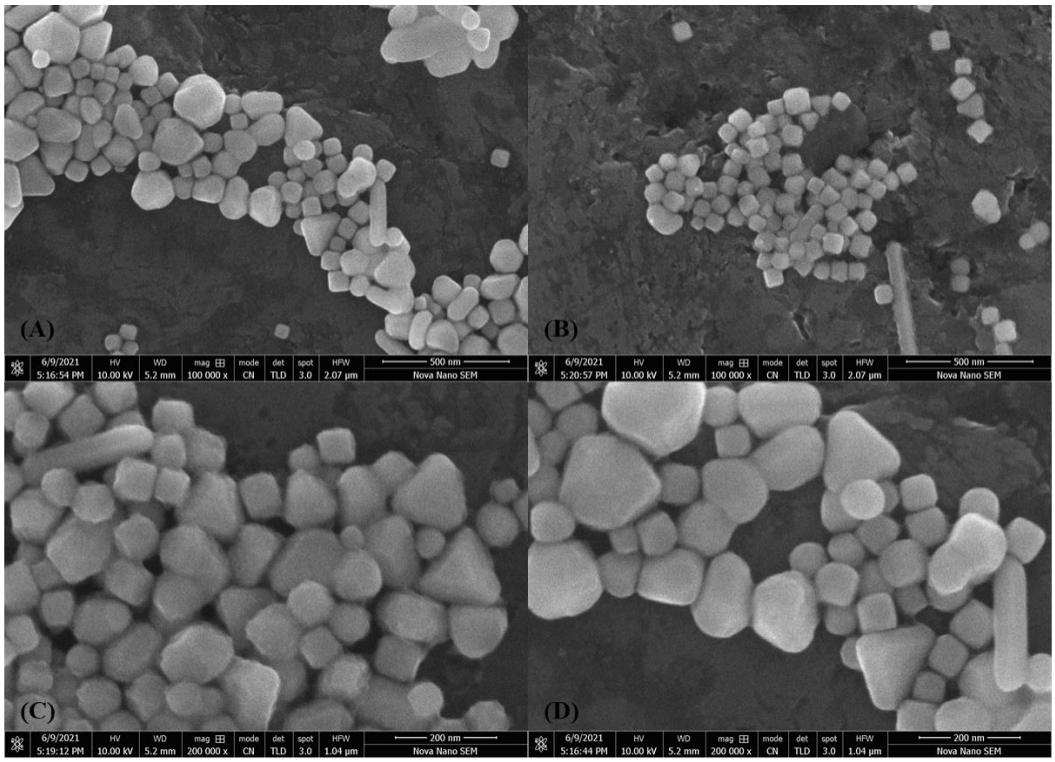

Figure 3 (A, B, C, D): S.E.M results of Silver NanoCubes synthesized using polyol synthesis shows a miscellany of shapes.

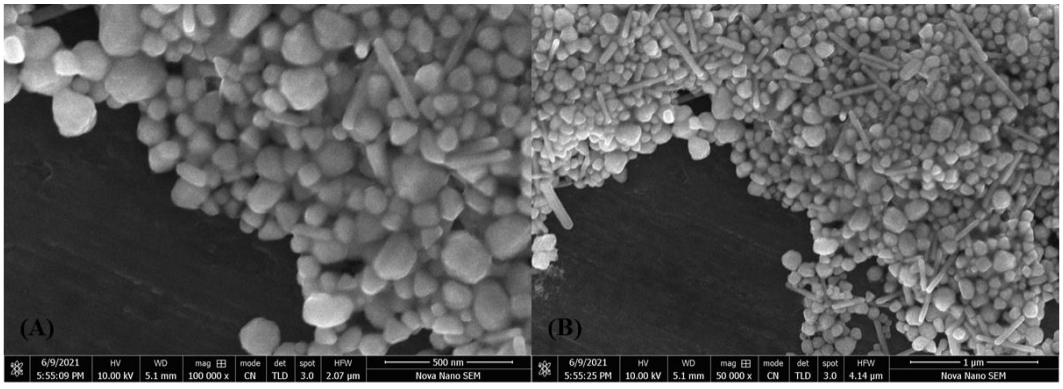

Figure 4 (A, B): S.E.M results of Silver NanoCubes synthesized using polyol synthesis shows a miscellany of shapes.

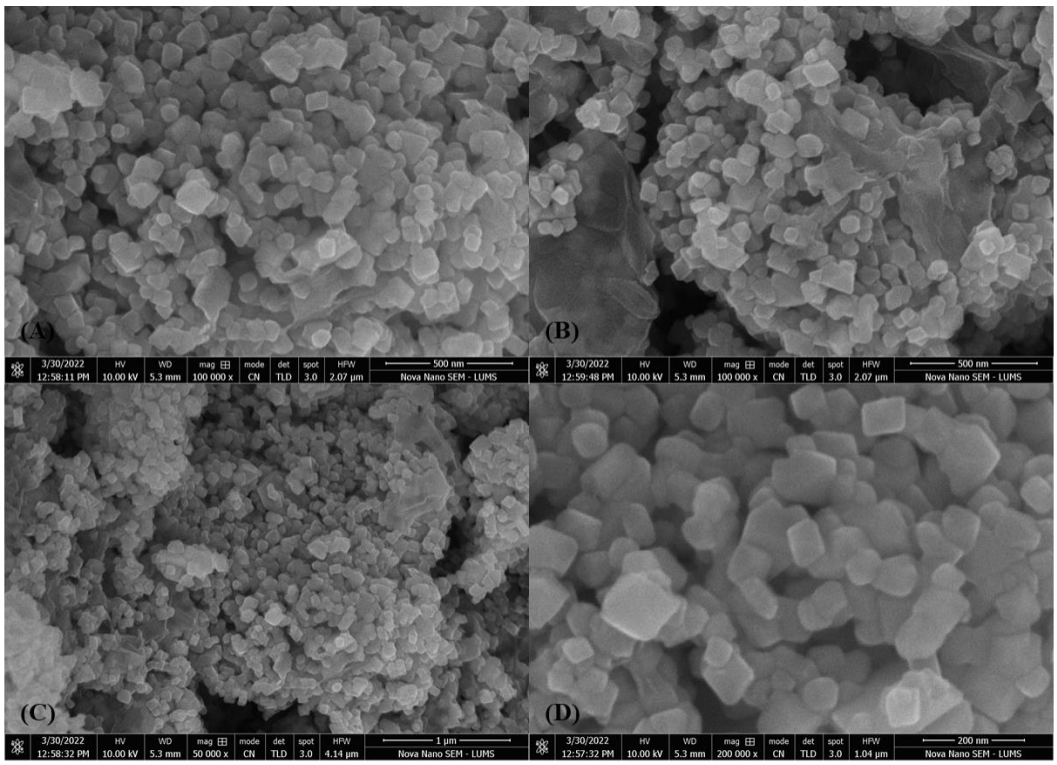

Figure 5 (A, B, C, D): S.E.M results of freshly prepared Silver NanoCubes synthesized using polyol synthesis shows a miscellany of shapes. Sample was subjected to S.E.M after storage for 1 month. No sonication before S.E.M was carried out.

3.2 Activities of Synthesized Nanocubes:

Synthesized NPs were subjected to various tests to assess their contribution in bioassays. An attempt was also made to analyze the effect of storage on the activity of the aged sample and the fresh sample.

AgNCs were examined for;

1. Antibacterial Activity
2. Antioxidant Activity

   a. FRAP Assay
   b. DPPH Assay
3. Hemolytic Activity
4. Anti-inflammatory Activity
5. Biofilm Inhibition Activity

Results of these assays are discussed in the following sections.

3.2.1 Antibacterial Activity:

Antibacterial activity of AgNCs was determined in triplicate using seven different bacterial strains viz., *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus pumilus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bordetella bronchiseptica*. Four different antibacterial drugs, Ampicillin (A), Ceftriaxone (B), Cefotaxime (C), and Meropenem (D), were used as standards or positive controls.

Silver metal has long been known to be innately antibacterial in nature [269] and its nanosized counterparts are no exception. Despite the fact that a comprehensive detailed account of the antibacterial mechanism of action of AgNPs is still not known, a few theories have been proposed. Potentially three different mechanisms are speculated to take place for an antibacterial effect. One, release of silver ions (Ag^+) from the NPs, uptake of said ions by cell, followed by disruption of the ATP cycle and DNA replication. Two, generation of Reactive Oxygen Species (ROS) by AgNPs and (Ag^+), and three, rupturing of cell membranes by AgNPs [270] to kill the bacteria.

Other factors have also been reported to play a role in the antimicrobial potential of AgNPs. Studies have shown that AgNPs with a smaller size [271] and spherical shape [272] display greater

antibacterial prowess. Generally, AgNPs with size smaller than 10 nm have been observed to release greater amounts of Ag^+ (due to larger surface area) which would impact the antibacterial effect. Relatively, in AgNPs with size greater than 10 nm a lower concentration of Ag^+ is released and in this case the NPs themselves carry out the antibacterial effect [273]. Furthermore, the surface modifications of AgNPs can cause a change in their antibacterial effect [274], generally, by stabilizing the AgNP and altering Ag^+ release. However, in doing so the antibacterial activity is in turn affected by the nature of the capping/modifying agent as well as the surrounding media [275]. It has been suggested that the AgNPs are toxic to both human and bacterial cells albeit with different mode of toxicity. It is believed that in human cells the entirety of the NP is taken up by the cell which leads to cytotoxicity whereas, in the case of bacteria the dissolved Ag^+ are believed to be the toxic components [276].

Our aim for this study was to assess whether AgNCs showed any antibacterial activity using different concentrations of the synthesized AgNCs as, to our knowledge, little is known regarding this particular shape. It is however known that the size and facets of AgNCs play an important role in deciding the magnitude of the antibacterial capability [195, 277]. The aged AgNCs sample showed no antibacterial activity in all seven species of the bacteria tested against any dilution of AgNCs (Figure 6). The positive control showed clear zones of inhibition indicating susceptibility of bacteria to the antibiotic and exhibiting no signs of antibiotic resistance. The combination discs, antibiotic + AgNCs, also showed a zone of inhibition with radius equal to that of positive control suggesting that the AgNCs had no synergistic or antagonistic contribution towards the effect of the antibiotic. It has been reported that the antibacterial activity of AgNPs lasts for 8-13 months [278] but we cannot ignore the size, shape, synthesis method and storage conditions of the AgNPs in their experiment.

The freshly synthesizes AgNCs sample showed no antibacterial activity against 6 out of seven species of bacteria used (Figure 7-12). *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus pumilus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bordetella bronchiseptica* all were resistant to 10 µl and 20 µl of the 10% dilution of fresh AgNCs tested. Antibiotic discs (5 µl loaded) showed clear zones of inhibition indicating no resistance to the drug. Zones of inhibition were observed in the antibiotic + AgNCs discs, but their size was equivalent to the one produced by positive control, indicating no synergistic or antagonistic effect of the AgNCs towards the antibiotic drug. Further tests using a 1% and 30% dilution of the AgNCs were carried out in an attempt to determine if a change in concentration of AgNCs would have any discrete effect on the antibacterial test. As evident from Figure 13, neither 1% nor 30% dilutions showed any antibacterial activity. These results hint at some form of resistance from the bacterial species against AgNCs.

Bacillus subtilis was the only strain that displayed sensitivity to AgNCs discs. Figure 14 exhibits the effect of 10 µl and 20 µl of 10% AgNCs. It also indicates that the discs containing 10 µl of the 10% dilution are more potent than 20 µl of 10% dilution. The antibiotic drugs showed clear zones of inhibition and the combination of drug + AgNCs had zones similar in size to those of positive control indicating no synergistic or antagonistic effect of AgNCs.

Keeping in mind *B. subtilis* was more susceptible to 10 µl of 10% AgNCs, the experiment was extended to see if a change in concentration would show any distinct change in the effect. AgNCs discs loaded with 10 µl of a 1% and 30% dilution of freshly prepared sample were made and tested. It is evident in Figure 15 that 1% dilution had a greater effect than 30% dilution of AgNCs.

Our results suggest that in general, the bacterial strains possess some kind of resistance to the antibacterial activity of Ag nano cubes. This may either be related to the shape and size of the nano

particles, release of the Ag^+ ions from the nano cubes, uptake of the Ag^+ ions/nano cubes through the bacterial cell wall/cell membrane or a combination of the various mechanisms. Bacteria that are susceptible to the AgNCs exhibit a better sensitivity at lower concentration of AgNCs rather than a higher concentration. This observation hints at some kind of uptake mechanism that becomes saturated at the higher concentrations. This hypothesis however needs to be further investigated through experimentation. We also conclude that over time AgNPs "age" which has a toll on their physicochemical and biological properties as described in some earlier studies [193]. We also hypothesize that different strains of bacteria have different modes of susceptibility for AgNCs. Nonetheless, a great deal of research is needed to fully understand and acknowledge the mechanism that takes place during antibacterial action of AgNCs.

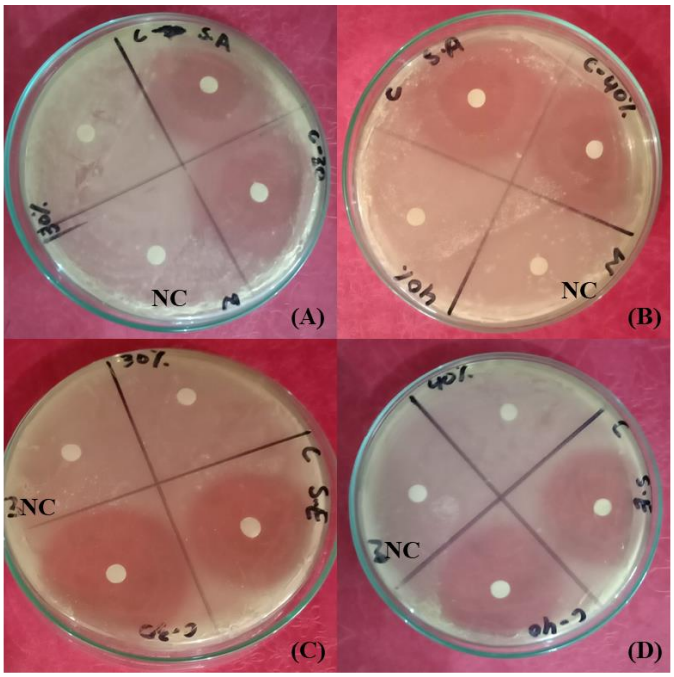

Figure 6 (A, B, C, D): Evaluation of the antibacterial activity of the aged AgNPs using *S. aureus* (Gram-positive) (A, B) and *S. epidermidis* (Gram-positive) (C, D). NC, the Negative Control disc, was placed in one corner of the plates. Antibiotic discs C (Cefotaxime) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl of a 30% and a 40% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

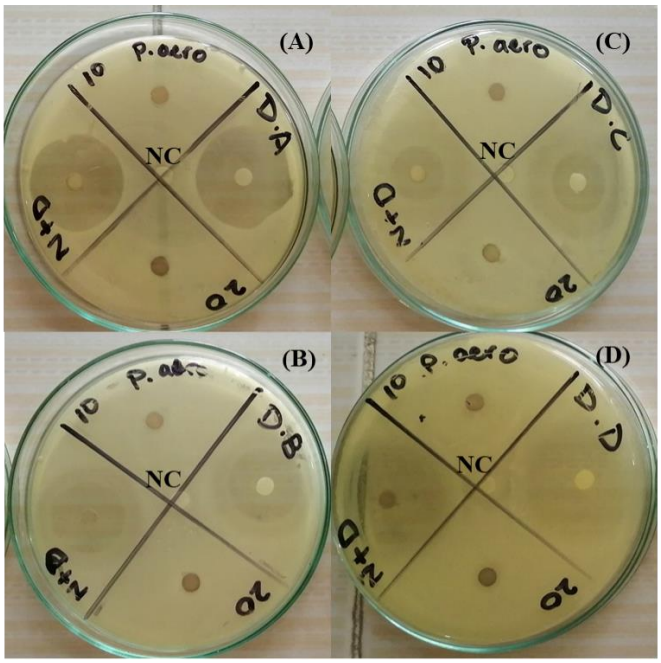

Figure 7 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *P. aeruginosa* a Gram-negative bacterium. NC, the Negative Control disc, was placed in the middle of the plate. Antibiotic discs DA (Ampicillin), DB (Ceftriaxone), DC (Cefotaxime) or DD (Meropenem) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl (10) and 20 µl (20) of a 10% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

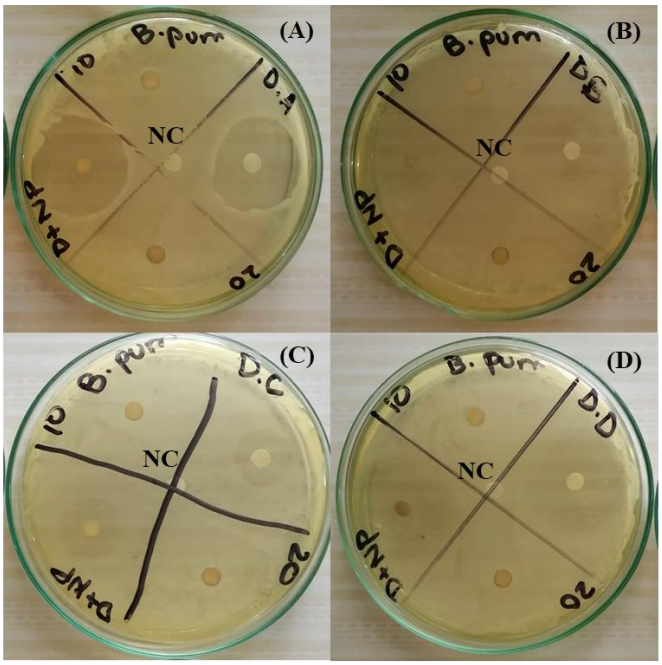

Figure 8 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *B. pumilus* a Gram-positive bacterium. NC, the Negative Control disc, was placed in the middle of the plate. Antibiotic discs DA (Ampicillin), DB (Ceftriaxone), DC (Cefotaxime) or DD (Meropenem) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl (10) and 20 µl (20) of a 10% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

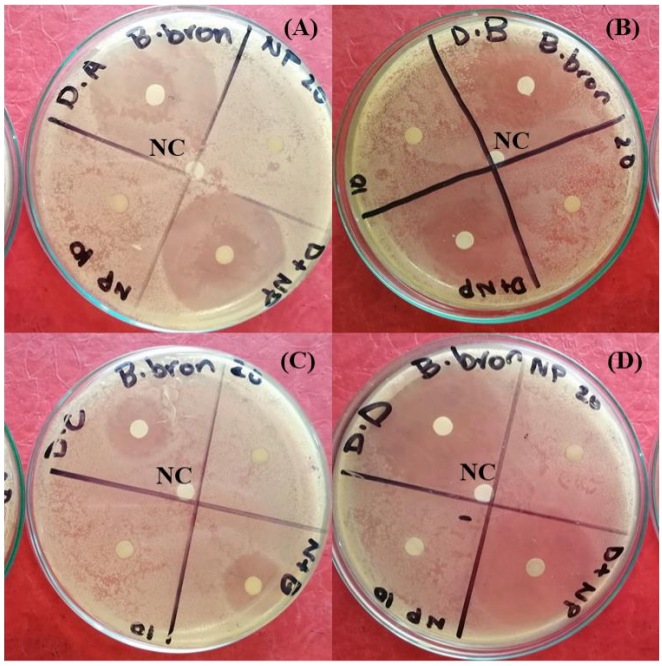

Figure 9 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *B. bronchiseptica* a Gram-negative bacterium. NC, the Negative Control disc, was placed in the middle of the plate. Antibiotic discs DA (Ampicillin), DB (Ceftriaxone), DC (Cefotaxime) or DD (Meropenem) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl (10) and 20 µl (20) of a 10% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

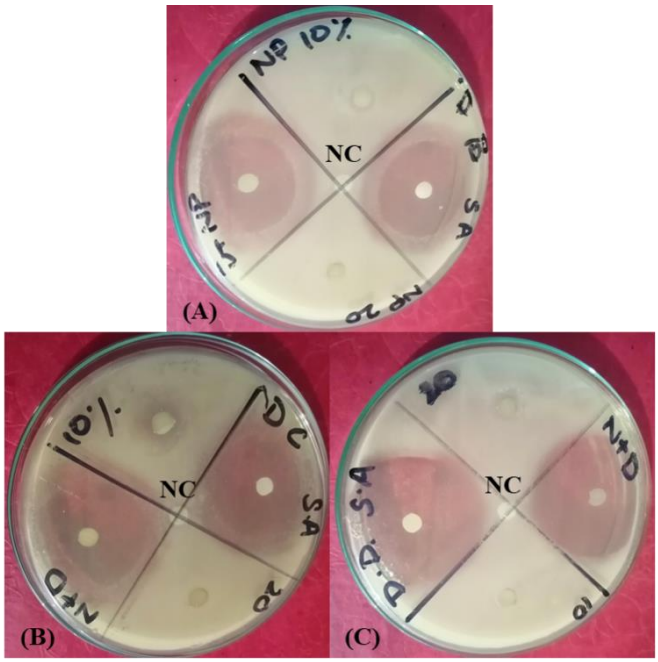

Figure 10 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *S. aureus* a Gram-positive bacterium. NC, the Negative Control disc, was placed in the middle of the plate. Antibiotic discs DB (Ceftriaxone), DC (Cefotaxime) or DD (Meropenem) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl (10) and 20 µl (20) of a 10% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

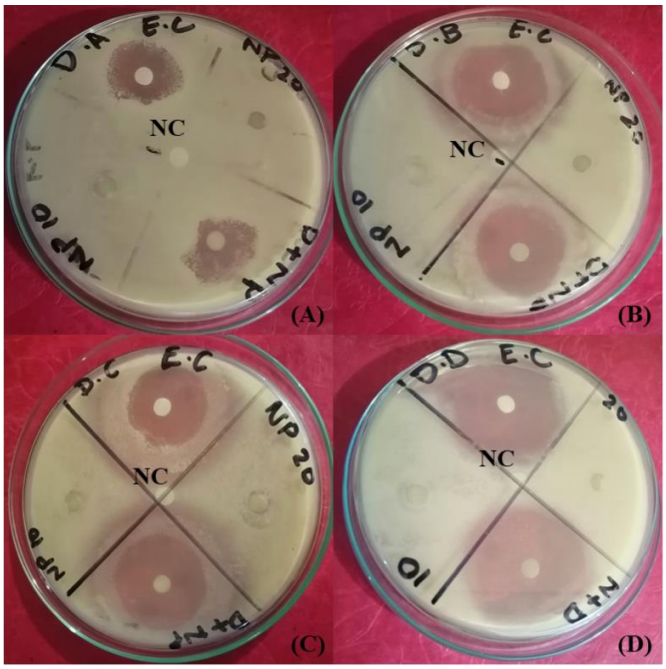

Figure 11 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *E. coli* a Gram-negative bacterium. NC, the Negative Control disc, was placed in the middle of the plate. Antibiotic discs DA (Ampicillin), DB (Ceftriaxone), DC (Cefotaxime) or DD (Meropenem) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl (10) and 20 µl (20) of a 10% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

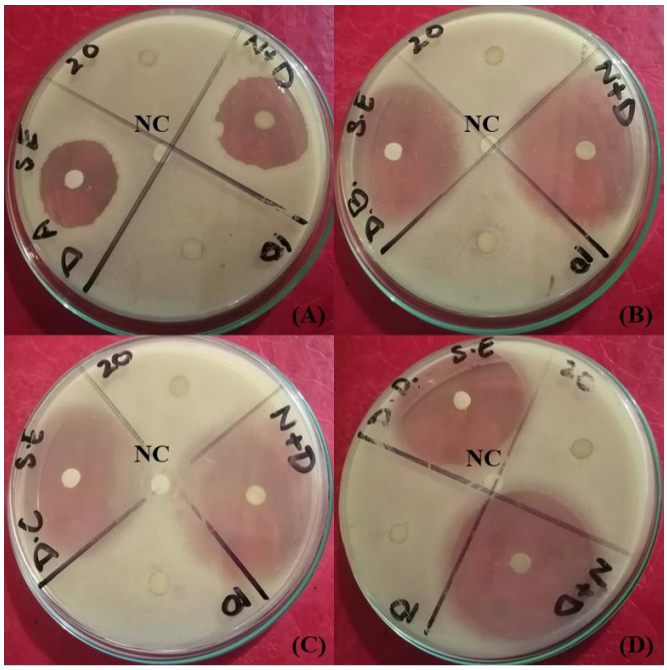

Figure 12 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *S. epidermidis* a Gram-positive bacterium. NC, the Negative Control disc, was placed in the middle of the plate. Antibiotic discs DA (Ampicillin), DB (Ceftriaxone), DC (Cefotaxime) or DD (Meropenem) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl (10) and 20 µl (20) of a 10% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

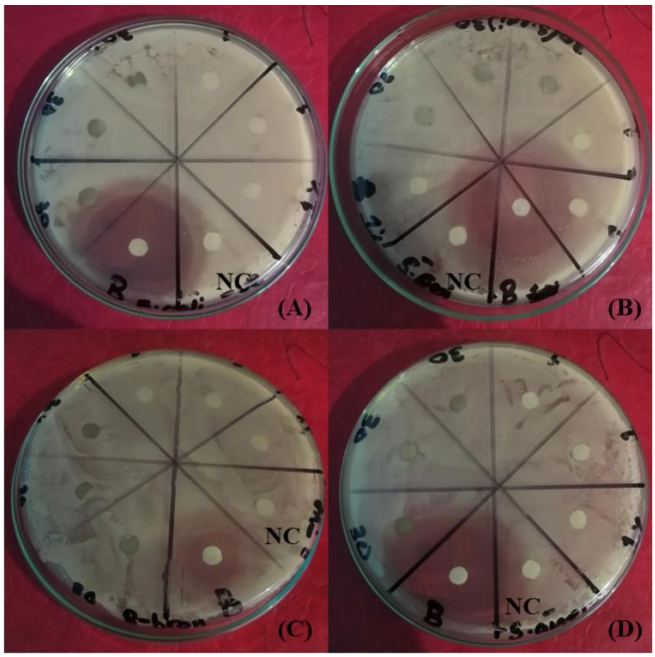

Figure 13 (A, B, C, D): Evaluation of the antibacterial activity of freshly prepared AgNPs using 6 bacterial strains *E. coli* (A), *S. epidermidis* (B), *B. bronchiseptica* (C), *S. aureus* (D), *B. pumilus, and P.* aeruginosa. NC, the Negative Control disc, was placed in one section of the plate. Antibiotic discs B (Ceftriaxone) carrying 5 µl 10 mg/ml of the antibiotic was used as the positive control. Discs carrying 10 µl of a 30% (30) and 1% (1) nanoparticles solution were used as test. To determine any antibacterial activity, these discs were used in triplicate.

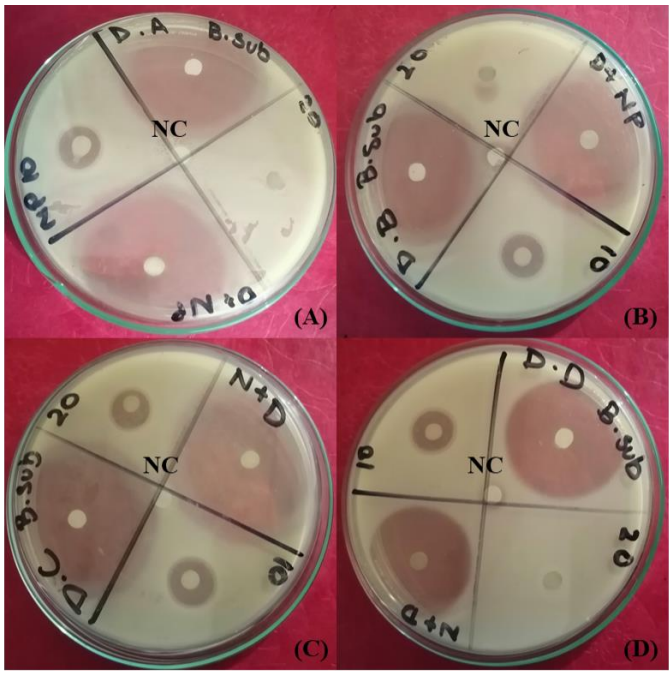

Figure 14 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *B. subtilis* a Gram-positive bacterium. NC, the Negative Control disc, was placed in the middle of the plate. Antibiotic discs DA (Ampicillin), DB (Ceftriaxone), DC (Cefotaxime) or DD (Meropenem) carrying 10 µl 10 mg/ml of the respective antibiotic were used as the positive controls. Discs carrying Nanoparticles present in 10 µl (10) and 20 µl (20) of a 10% solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effect of the NPs.

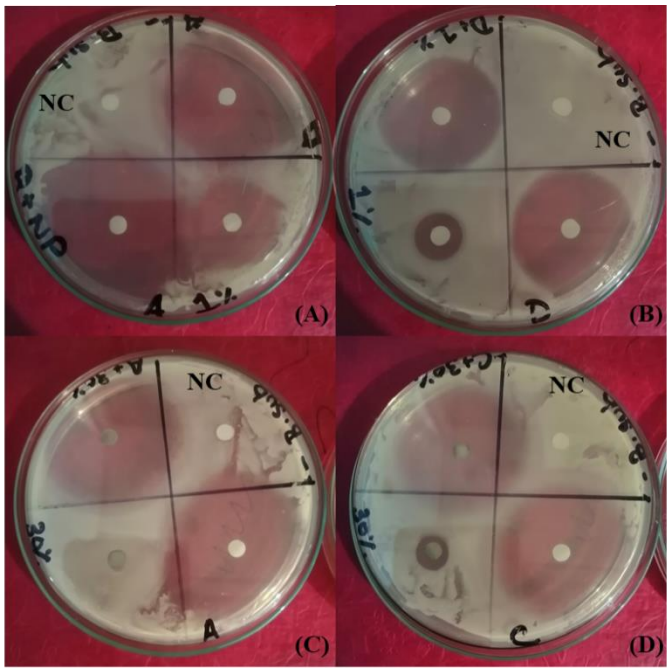

Figure 15 (A, B, C, D): Evaluation of the antibacterial activity of the freshly synthesized AgNPs using *B. subtilis*. NC, the Negative Control disc was placed in the middle of the plate. Antibiotic discs A (Ampicillin), B (Ceftriaxone), C (Cefotaxime), D (Meropenem) carrying 5 µl 10 mg/ml of the respective antibiotics were used as the positive controls. Nanoparticle discs carrying 10 µl of 1% (A,B) and 30% (C,D) solution were used as the test. In addition, N+D discs carrying NPs and the respective antibiotic were used to evaluate any synergistic or antagonistic effete of the NPs.

3.2.2 Antioxidant Activity:

Countless AgNPs synthesized via green synthesis method have been evaluated for their antioxidant activity [279, 280] and have shown excellent antioxidant activity. Comparative studies of the antioxidant potential between green synthesized and chemically synthesized AgNPs highlight that the green synthesized AgNPs possess far greater antioxidant capabilities as compared to AgNP produced by chemical synthesis [281, 282]. This increase in antioxidant activity displayed by green synthesized AgNP is thought to be a result of surface modifications of the AgNP caused by phytochemicals. Interestingly, the published literature lacks any reports regarding antioxidant potential of the AgNCs or the mechanisms involved therein. Ours is therefore among the first reports regarding the antioxidant potential of AgNC, if any. Antioxidant potential of our AgNCs was determined using two different and very popular antioxidant assays, i.e, FRAP assay and the DPPH assay.

1. FRAP Assay

The FRAP antioxidant assay was performed in triplicate to determine the antioxidant activity (if any) of AgNCs. Table 6 shows the results obtained from a 40% dilution of the aged AgNCs. The absorbance values of the 40% dilution were parallel to the Negative control suggesting no antioxidant activity. However, a notable antioxidant activity pattern was observed when the 1%, 10% and 20% dilutions of the freshly prepared AgNCs were examined for their antioxidant activities. In Figure 16, we can see the distinct color changes observed for negative control, positive control and the three different dilutions of AgNCs tested. Table 7 displays the absorbance values of these samples at 700 nm. There is a clear increase in the antioxidant activity of AgNCs as the samples become more concentrated.

As such, a highly concentrated sample such as a 40% dilution, should have shown a high value of antioxidant activity. The absence of antioxidant activity in the aged sample is vindicated by the age of the sample. Since it is expected that the Ag^{+} ions trapped inside the AgNCs would serve as the active antioxidant component, we postulate that either these ions get completely lost due to reduction in the aged samples or they get trapped in the nanocubes debris of the aged particles. On the contrary, the fresh nanocubes not only retain and protect these active ingredients they also release these ions in the assay conditions effectively.

Table 6: Antioxidant activity of the aged NPs using FRAP assay.

Sample	Absorbance at 700 nm	Absorbance at 700 nm	Absorbance at 700 nm
Negative (Water)	0.373	0.329	0.305
Ascorbic Acid	overflow	overflow	Overflow
Aged sample 40%	0.372	0.406	0.300

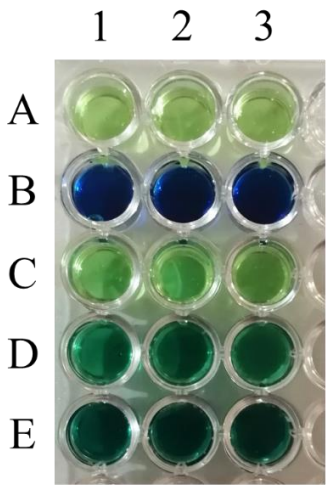

Figure 16: Evaluation of the antioxidant potential of the nanoparticles using FRAP assay. Lane A (Well 1-3) Negative Control. Lane B (Well 1-3) Positive Control. Lane C (Well 1-3) 1% solution of freshly prepared NPs. Lane D (Well 1-3) 10% solution of freshly prepared NPs. Lane E (Well 1-3) 20% solution of freshly prepared NPs.

Table 7: Antioxidant activity of the freshly synthesized NPs using FRAP assay.

Sample	Absorbance at 700 nm	Absorbance at 700 nm	Absorbance at 700 nm
Water (negative)	0.346	0.316	0.326
Ascorbic Acid	overflow	Overflow	overflow
1% (NS)	0.280	0.320	0.293
10% (NS)	0.958	0.967	1.012
20% (NS)	1.894	1.937	1.885

2. DPPH Assay:

Additionally, DPPH assay was also performed on both AgNC samples to assess their antioxidant prowess. Table 8 assuredly demonstrates the same results as indicated by the FRAP assay. The aged sample, regardless of the concentration, showed little to no antioxidant activity whereas the freshly prepared sample showed high levels of antioxidant activity with the increasing concentration of the AgNC sample. Just as a comparison the 10% dilution of the old sample gave a value of 2.1 which is less than the value of 2.7 presented by even the 1% diluted fresh sample.

Table 8: Antioxidant activity of the AgNPs (aged and fresh) using DPPH assay*.

Sample	Concentration (%)	Antioxidant activity (%)
1. Aged Sample	10	2.1
	20	1.9
	30	2.4
	40	3.2
2. Fresh Sample	1	2.7
	10	21.19
	20	45.38
Quercetin (0.5 mM)		85.65±0.15
Propyl gallate (0.5 mM)		80.71±0.14

***Four concentrations of the aged sample were used (10%, 20%, 30% and 40%). Three concentrations of the fresh sample (1%, 10% and 20%) were used.**

Our assessment of the antioxidant activity of AgNCs synthesized via polyol method have displayed stimulating results. The aged AgNCs, as in the case of antibacterial activity, have lost their antioxidant activity as well. The freshly prepared AgNCs sample has shown antioxidant activity directly proportional to the increasing concentration used. As little work has been done to evaluate the antioxidant activity of AgNCs, we can confidently say that the shape of the NP helps to protect the antioxidant activity of the active ingredient i.e., Ag^{+} ions although further studies need to be carried out in order to understand and elucidate the mechanisms associated with the change in the extent of antioxidant activity.

3.2.3 Hemolytic Activity:

Taking into consideration the future use of AgNPs in medicine, it is inevitable that these particles will be coming into direct contact with erythrocytes. The hemolytic activity of AgNCs was therefore performed to determine their toxicity, if any, towards Red Blood Cells (RBC). The hemolytic assay performed on our AgNCs showed little to no hemolytic activity, with negligible deviation suggesting some kind of inertness towards hemolysis (Table 9). This estimation was conducted in reference to 0.01% Triton-X 100, the positive control, that exhibited 99% hemolytic activity.

Previous studies conducted on AgNPs have suggested that the hemolytic activity of AgNPs is size dependent. Smaller AgNPs, with size 15 nm, were observed to be more hemolytic in nature. The RBC damaging property has been attributed to the direct interaction of the nanoparticles with the RBCs [255]. Another study compared the cytotoxic effect of different shapes of AgNPs on RBCs. Between spherical and wire shaped AgNPs of different sizes, the size and dose of the particles proved to be a greater contributing factor in the hemolytic activity rather than the shape [283]. As a result, AgNPs of a specific size, preferably a size large enough to exhibit inertness towards

RBCs, present great potential for use inside the body. The lack of hemolytic activity displayed by our AgNCs can be ascribed to the size of the NPs and it appears that these cubes are safe to use as carriers of a drug for targeted drug delivery. This preliminary observation, however, needs to be investigated in detail through further experimentation.

Table 9: Hemolytic activity of the AgNCs.

Sample	% Hemolysis	Standard Deviation
AgNCs (20 µl)	4.72813238770686	0.057888
0.01% Triton-X 100 (20 µl)	99.05437352	0.06477

3.2.4 Anti-inflammatory Activity:

The beneficial properties, if any, of AgNPs for human health were further evaluated by conducting anti-inflammatory test. This activity is performed to determine if a substance reduces inflammation and as such encourage the healing process. Our results, shown in Table 10, indicate that AgNCs possess a relatively strong anti-inflammatory activity. While under our assay conditions the positive control, Diclofenac sodium, causes 74% reduction in the inflammation, our test material, the AgNCs, remarkably reduce inflammation by 62%. Thus, these silver nanocubes have the potential of being used as anti-inflammatory agents.

Through a study in porcine models, nanocrystalline silver has been demonstrated to possess remarkable anti-inflammatory properties [258]. This study showed an increase in inflammatory cell apoptosis, decrease in proinflammatory cytokines expression and a decrease in gelatinase

activity, all presumed to be directly caused by nanocrystalline silver. Similarly, another study has revealed the anti-inflammatory characteristic of green synthesized AgNPs both *in vitro* and *in vivo*, highlighting in both cases decrease in levels of cytokines, in addition to advocating their potential use in the treatment of psoriasis vulgaris skin lesions [259]. In accordance with these findings, AgNPs/nanocrystalline silver has been used extensively in wound dressings to facilitate healing of the wound [284, 285]. Further experimentation is needed to ascertain whether AgNCs are more potent anti-inflammatory mediators compared to other Ag nanomaterials.

Table 10: Anti-inflammatory activity of the AgNCs.

Sample	% Anti-inflammatory activity	Standard Deviation
AgNCs (20 µl)	62.4203821656051	0.086767
Diclofenac sodium (20 uL) conc. 10 mg/mL	74.37	0.05698

3.2.5 Biofilm Inhibition:

Bacterial species are among the most adaptable life forms on earth. They can exist as individual cellular structures or, as a survival mechanism, these micro-organisms can form complex 3-dimensional multilayered structures referred to as biofilms on biological or non-biological surfaces by secreting various extracellular polymeric substances (EPS) [286]. Biofilms can be a result of a single species as well as a mixture of different species of bacteria. The type of bacterial strain(s) and different environmental factors play a role in determining the varying physicochemical properties of these biofilms [287]. Following the irreversible attachment and colonization of

bacterial species on a solid surface, rapid bacterial cell growth takes place and EPS is produced resulting in the formation of a biofilm [288]. These biofilms are a cause of various chronic diseases of the auditory system [289], the circulatory system [290], the digestive system [291], the integumentary system [292], the reproductive system [293], the respiratory system [294], and the urinary system [295]. They may even lead to some forms of cancers [296]. Biofilm inhibitors therefore have a beneficial potential for the treatment of bacterial infections. With the increase in bacterial resistance towards antibiotics, lack of availability of novel antimicrobial chemicals and the protective EPS layer provided by the biofilms, researchers have directed their attention towards nanotechnology and its possible therapeutic applications against microbial infections [297-302]. Regardless of these variations, all biofilms share some common properties, the most familiar of which is there tendency to interact, specifically or non-specifically, with living or non-living surfaces in an effort to become sessile [303]. Metallic nanoparticles show great promise in altering the metabolic activity of bacteria [304] and hence hold potential as a form of treatment of bacterial diseases. Amongst these, AgNPs particularly have been seen to enter biofilms and suppress the genes that allow for biofilm formation, consequently preventing biofilm formation [305]. This antibacterial property is generally considered to be a result of released Ag ions.

During our experiments performed on *Bacillus subtilis*, our AgNCs gave a 43.6% biofilm inhibition with a standard deviation of ±0.04 (Table 11). These results substantiate previous findings that AgNPs are good inhibitors of biofilm formation and suggest that the shape of NPs has little effect on their inhibitory activity. This may be because the main element responsible for biofilm inhibition is the Ag ions and not the AgNPs itself.

Table 11: Biofilm inhibition of the AgNCs performed on Bacillus subtilis.

Sample	% Biofilm Inhibition	Standard Deviation
AgNCs (100 µl)	43.6363636363636	0.04532
Ciprofloxacin (100 uL) conc. 10 mg/mL	61.8181818181818	0.05643

Conclusion

The aim of the present investigation was to synthesize AgNCs and assess their potential for use in health biotechnology. AgNCs were successfully synthesized using polyol reduction method and characterized via SEM. They were assessed for a number of biological properties including antibacterial prowess, antioxidant activity, hemolytic activity, anti-inflammatory activity and biofilm inhibition activity. Overall our results are analogous to previous studies performed on AgNPs¸ mainly because the biological properties of AgNPs are affected mostly by their size and the ability to release Ag^+ rather than the shape of the particle [306].

In some cases, such as in hemolytic assays, a larger size for AgNPs is preferred as it renders them inert towards RBCs. Other activities such as anti-oxidant and anti-inflammatory activities of AgNCs require comprehensive studies to be carried out before any further conclusions can be drawn. According to the literature the antibacterial activity is the most extensively studied property of the AgNPs, but surprisingly the exact mechanism of action that takes place is still not known. Undeniably, AgNPs are highly antibacterial in nature, but even the slightest alteration in size,

shape, surface modification, and even NP facets appear to produce a drastic difference in the magnitude of antibacterial activity. Similarly, the ability to inhibit biofilm synthesis is an extensively studied parameter, and it is mainly affected by the ability of the AgNPs to release Ag^+. We know from previous studies that AgNPs with smaller size have greater biofilm inhibiting ability as the larger surface area allows for greater Ag^+ release.

Our results indicate that the AgNCs possess great potential for use in medicine. Nonetheless there is great need for further experimentation to elucidate in detail exact mechanisms of the observed characteristics and validation of their beneficial properties over AgNPs in other shapes. It is therefore suggested that studies be planned to work out these mechanism in details. AgNPs be prepared in different shapes and tested for their biological activities. We can ask that if the AgNPs are formed after reduction of the Ag^+ ions into metallic silver, then how do these NPs exert their activities through the release of Ag^+ ions as suggested by several workers [307]. It has been proposed that the silver NPs undergo an oxidative dissolution generating Ag^+ ions, a process that may either involve H_2O_2 or Oxygen. If true, the AgNPs would be expected to be gradually converted back into ionic silver and lose their activities. Our results coincide with this speculation as the newly synthesized and the stored old NPs vary in their characteristics significantly. The NP's size to shape ratio is another interesting parameter that needs to be studied thoroughly. Is it possible to prepare NPs having a specific shape in varying sizes and if so, what will be the effect(s) of these size variations on the properties of these NPs? Answers to such questions can provide us very important information about the NPs in general and AgNPs and AgNCs, in particular. The information thus obtained can then be explored for the potential use of these NPs in health biotechnology to avail their beneficial effects for humanity.

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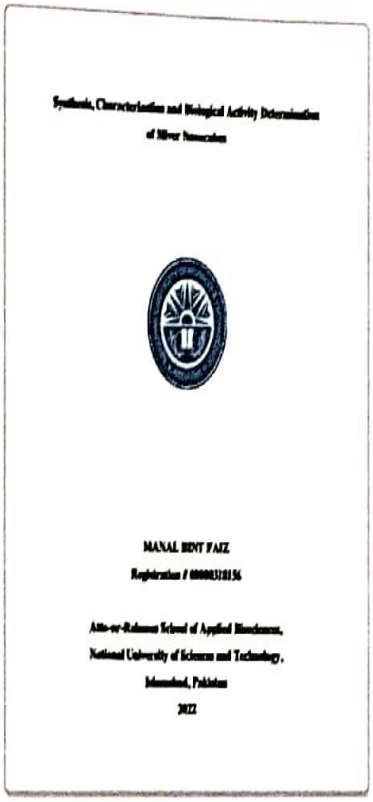

Synthesis, Characterization and Biological Activity Determination of Silver Nanoscales

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