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Synthesis of poly(amidoamine)-dendrimer-silver nanoparticles composite for application as bactericides

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ABSTRACT

As awareness of sanitation, disease transmission factors and personal protection increases, researchers have focused on developing materials with antibacterial properties. Silver-PAMAM-dendrimer has unique antibacterial properties that make it an ideal candidate for medical applications. In this study, poly (amidoamine) (PAMAM) dendrimers from Generations 0.5- 4.0 were synthesized using divergent methods. Silver was combined with PAMAM dendrimers from the Generation 2.0, 3.0 and 4.0 to form colloidal solutions. The antimicrobial capabilities of Silver-PAMAM-dendrimer (Generation 2.0) were tested against four types of gram positive and negative bacteria. Its bactericidal activities were revealed for all four types of bacteria that could not grow in samples containing 600 ppm of Silver-PAMAM-dendrimer (Generation 2.0).

Keywords: *Bactericidal activity, PAMAM-Dendrimer, Silver nanoparticles, Divergent method*

1. INTRODUCTION

Since ancient times, silver has been recognized as a substance with antiseptic properties. In both its metal and ionic form, silver exhibits strong microbicidal properties towards a broad range of microorganisms [1-3]. The antimicrobial property of silver is one of the best compared to other metals, which can be ranked as follows in terms of their antimicrobial properties:

$$Ag>Hg>Cu>Cd>Cr>Pb>Co>Au>Zn>Fe>Mn>Mo>Sn$$

The antibacterial function of silver nanoparticles is similar to that of silver ions [4]. As a result, a growing interest in the synthesis and study of substances containing silver has developed. One promising nanoparticulate material called Polyamidoamine (PAMAM) dendrimers is an excellent template for metals and it has attracted attention because of its potential for a wide range of biomedical and industrial applications [5].

Dendrimers are globular, nano-scaled, highly branched, and regular polymer macromolecules with low toxicity. Unlike polymers, dendrimers have a high degree of molecular uniformity and specific sizes. Their multifunctional surface groups can be linked with other chemical moieties to modify the surface properties of the dendrimers. Additionally, their high solubility makes them very different from linear polymers. Their high solubility can be seen for several types of dendrimers by comparing them with a corresponding linear polymer. This is especially true for polyphenylene, polyaryleter, and polyamide dendrimers. Differences in the solubility between branched and linear compounds can be of several orders of magnitude, up to 106 times in some cases. Even higher generations of dendrimers (Generation 10 or 12) remain soluble despite molecular weights close to one or even several thousand grams.

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One of the distinct properties of half-generation dendrimers, as opposed to full generation dendrimers, is their surface activity. The surface activity of half-generation dendrimers creates a foamy solution in water media [6]. Half-generation dendrimers have lower intrinsic viscosities than corresponding full-generation dendrimers, which is a result of the increased aggregation of amine-terminations caused by intermolecular hydrogen bonding.

Early generations of dendrimers tend to exist as relative open forms providing reactive sites on the periphery of the dendrimer. Thus, in theory, the preparation of metal nanoparticles formed inside the early generation dendrimers is more difficult than it is for later generations of dendrimers. Despite this potential drawback, early generations of dendrimers have many advantages, such as being easy to synthesize, making them worth preparing.

The structure and chemical properties of earlier generations of dendrimers are different from linear polymers with random-coil structures [7]. Their structure may be important, but a solvent incapable of dissolving a dendrimer is highly dependent on the type of terminal groups in the dendrimer. For example, PAMAM dendrimers are water soluble substances and are considered to be critical for biological applications [8]. Another important property of a PAMAM dendrimer is its tendency to form cationic structures under physiological conditions. Under these conditions, the primary amines on the surface of the dendrimer become readily protonate. This creates a polycationic dendrimer with ammonium terminal groups, which increases the alkalinity of a solution.

The properties listed above encouraged us to combine silver particles in a dendrimer for application as a bactericide with minimum toxicity. The goal of this study was to synthesze a poly (amidoamine) (PAMAM) dendrimers/silver composite compound and to investigate its ability to release silver ions inside bacterial cells.

2. EXPERIMENTAL SECTION

2.1. Preparation of PAMAM-Dendrimer-Silver. The synthesis of ethylenediamine (EDA) -core PAMAM dendrimers consists of two stages. The first stage involves adding a primary amine, ethylenediamine (EDA) (not less than 99% purity) to methyl acrylate (MA) (not less than 99% purity). The second stage is the amidation of the formed multiester with EDA. Repeating this two stage procedure leads to the formation of higher generation dendrimers.

2.1.1. First Stage: the synthesis of ester-terminated dendrimer (half generation, G 0.5). The reaction media was prepared in two parts. Parts A and B consisted of dissolving EDA in methanol and MA in separate containers. To start the reaction, these two solutions were combined to create a mixture with a molar ratio of 1:4 (EDA/MA). The solution in Part A was added drop-by-drop to the solution containing Part B at 27°C. The mixture was stirred continuously for 72 hours and it was kept 27°C. Finally, the excess reactant, MA and solvent were removed by evaporation using a rotary evaporator at 70°C.

2.1.2. Second Stage: the synthesis of amine-terminated dendrimer (full generation, G 1.0). As in the first stage, the second stage was also in two parts. In Part A, half generation PAMAM prepared in Stage 1, was dissolved in methanol. In Part B, EDA was dissolved in the same solvent. Next, the multi-ester solution from Part A was gradually added to the stirred EDA solution from Part B. Then the mixture was stirred and allowed to react for 72 hours at 27°C. Excess EDA was removed using an azeotropic mixture of toluene and methanol (9:1 v/v) and the remaining toluene was then removed by azeotropic distillation with methanol. Finally, the excess methanol was removed by an evaporation process in a rotary evaporator at 70°C.

2.1.3. Synthesis of PAMAM-Dendrimer-Silver composite. About 15 mL of Argentum nitrate (AgNO3) solution (25ppm, 50ppm or 100ppm), 0.365 g of PAMAM-dendrimer solution (10 wt %

MeOH solution) (from Generation 2.0, 3.0 or 4.0) and 0.6 mL of MeOH were mixed together to produce a reaction medium. Then, $NaBH_4$ (15mg) was dissolved in mixture of methanol (2mL) and water (1:2 in volume) followed by the slow addition of the entire $NaBH_4$ solution while stirring. The reaction mixture turned a yellow- brown colour within a few seconds after the addition of the first drop of the $NaBH_4$ solution. The solution was stirred for 2 hours to complete the reaction. Finally a yellow or brown colloidal solution of PAMAM-dendrimer in a MeOH/water (1:2 in volume) mixture was obtained.

2.2. Antibacterial Activity Assay. A qualitative antibacterial activity assay (disk diffusion) method was conducted to determine the antimicrobial properties of silver-PAMAM-dendrimer nanoparticles using the following standard test organisms: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis.*

2.2.1. Disk Diffusion Method. Prior to starting this test, all the materials including the flask, filter paper disks, and pipettes were sterilized using an autoclave for 15 minutes at 121°C. The nutrient agar was distributed in Petri plates and inoculated with bacteria. Each bacterium was obtained from liquid cultures developed for 24 hours at 30°C, with shaking except for the *E. coli* grown at 37°C. After 24 hours, the bacterial cultures (0.1mL) were transferred to nutrient agar plates and spread using special glass rods. Next, filter paper disks containing either 0.01mL of silver-PAMAM dendrimers or a control solution were spotted on each plate. The inhibition zone was monitored after the plates were incubated for 24 hours at 30°C (37 °C for *E. coli*).

2.3. Fourier Transform Infrared Spectroscopy(FTIR). The molecular structure changes in PAMAM dendrimer G 0.5-4.0 were categorized using FTIR spectrometer (Perkin Elmer) analysis. IR spectra were obtained with a spectrum of 100 series using a transformation of 20 scans with a spectral resolution of 4 cm^{-1}. FTIR spectra were collected in the mid-infrared region between 4000 and 500 cm^{-1}. Spectra were acquired using an air-background correction and related software.

2.4. UV-Visible Spectroscopy. In this study, a Perkin Elmer Lambda 25 UV-VIS Spectrophotometer was used in order to measure absorption. To determine the degree of absorption, each sample (about 3mL) was placed in the quartz cell holder which was then placed and locked in the analyser compartment. The sample was scanned using wavelengths in the range of 550- 300nm.

2.5. Fluorescence Spectroscopy. In order to obtain a fluorescence spectrum for colloidal Ag-PAMAM, a fluorescence spectrometer (FLS920 model) was used. The sample was scanned using wavelengths in the range of 700- 420 nm.

3. RESULTS SECTION

3.1. Formation of Silver-PAMAM-Dendrimer. During the reduction of the silver nitrate solution containing PAMAM-dendrimers with sodium borohydride, the solution turned yellow-brown and there was a corresponding increase in the Ag concentration of the solution. The equations for the reduction process of silver ions, the oxidation, and hydrolysis of sodium borohydride are shown below:

$Ag^+ + e^- \leftrightarrow Ag^0$

Oxidation of sodium borohydride:

$BH_4^- + 3H_2O \leftrightarrow H_3BO_3 + 7H^+ + 8e^-$

Hydrolysis of sodium borohydride:

$BH_4^- + 3H_2O \leftrightarrow 2H^+ + BO_3^{3-} + 4H_2$

The change in the colour of the solution from clear to yellow or brown indicated the formation of Ag (0) particles. After stirring for 2 hours, the solution turned light yellow. This phenomenon can be explained by UV-Visible characterization. All PAMAM-Dendrimers-Silver composite from Generations 2.0, 3.0 and 4.0 showed similar UV-Vis spectra. Before the reduction process took place, there were no peaks in the visible light spectrum. However, after the reduction process, maximum peaks were observed at around ~ 410nm but they shifted slightly to correspond with the concentration of Ag. Observed shifts of this type are often explained as a gradual decrease in the diameter of the initially formed fluffy particles composed of smaller clusters. According to Stofik *et al.*, with a simultaneous increase in the plasmon peak intensity, the cluster develops into smaller particles [9]. Figure 1 shows the UV-Vis spectra of a Generation 4 dendrimer after it was subject to a 2 hour reduction process.

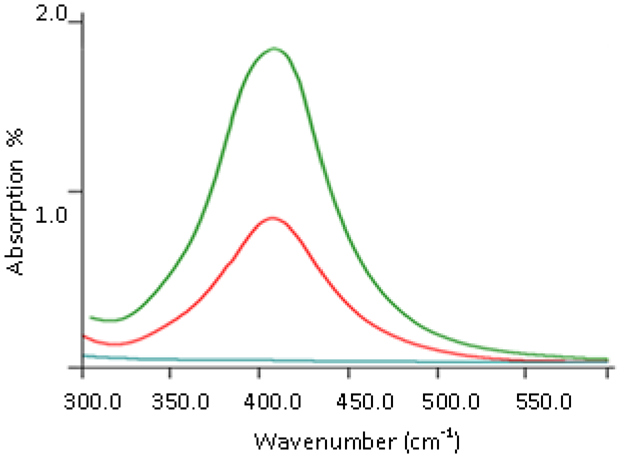

Figure 1: UV-Vis spectra of composite after reduction of silver nitrate

In this type of reduction process, there are usually two possible silver nanoparticles formations that can occur in the PAMAM dendrimer. Shen and Shi reported that the formation of dendrimer-encapsulated nanoparticles (DENPs) occur using fast reduction techniques and nucleation chemistry. The size of DENPs particles is normally smaller than 5nm [10]. The two stages in the creation of DENPs are a ligand/metal-ion interaction and the pre-organization of metal ions by the dendrimer host using other types of the weaker forces. The formation of dendrimer-metal complexes is in dynamic equilibrium with the template and metal ions and it allows an equal distribution of the metal ions between all equivalent ligands in the dendrimer molecules and diffusion. Thus, it provides a homogenous distribution of metal ions within the dendrimer template. Subsequently, the formation of DENPs is caused by the reduction of pre-organized metal ions.

When metal or other inorganic nanoparticles (NP) are surrounded with multiple dendrimer molecules, the resulting nanostructure is referred to as a dendrimer stabilized nanoparticles (DSNPs). Metal DSNPs are usually formed under mild reduction conditions that assist the slow nucleation of the particles. The formation of DSNPs also depends on the structure of the dendrimers used. If earlier generations of dendrimers are used as template, then the reduction of fats and nucleation can still afford the formation of DSNPs instead of DENPs because the limited terminal amines and open structure cannot entrap metal NPs inside the dendrimer. Mechanistic studies show that dendrimer terminal amines are extremely effective in the stabilization of metal NPs. Although many reports show that DSNPs can be formed using a simple thermo treatment [11], UV-irradiation [12], or laser ablation [13] of the dendrimer-metal complex, the chemical reduction process is still the technique favoured to synthesis DSNPs. Therefore, in this study, silver nanoparticles investigated were DSNPs. Figure 3 illustrates two types of silver nanoparticle formations in a PAMAM dendrimer.

3.2. FTIR Spectroscopy. Figure 2 (a) and (b) presents the FTIR spectra of the PAMAM-dendrimer. The absorption bands at 3400 – 3350 cm-1 were characteristics of O-H and N-H stretching frequencies. The bands in the range of 2950 - 2835 cm-1 were attributed to the C-H stretching vibrations of the CH2 and CH3 groups. A comparison of the FTIR spectra of full generations and half generations of PAMAM-dendrimers demonstrate absorption peaks around 1735 cm-1 and 1745 cm-1, which indicated the formation of ester bonds (-COCH3) in the half generations of PAMAM-dendrimers. In the amidation reactions, EDA or amine terminated groups reacted with the ester groups on half generations to produce amide (-CONH-) and amine surface groups in the full generation. The two new IR bands at ~1635 and ~1560 cm-1 were characteristic of these primary and secondary amides groups. The disappearance of ester groups at 1735 -1745 cm-1 indicated that the amidation step was completed.

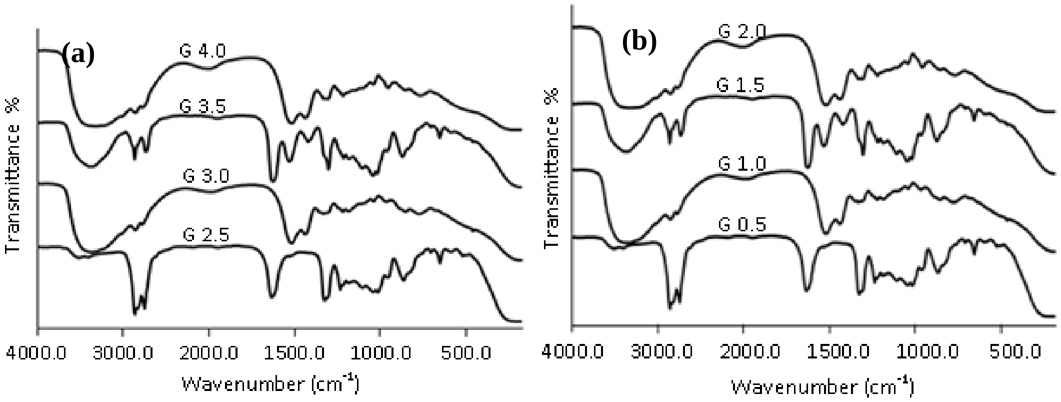

Figure 2: FTIR spectra of dendrimers, a: G 0.5-2.0, b: G2.5 4.0

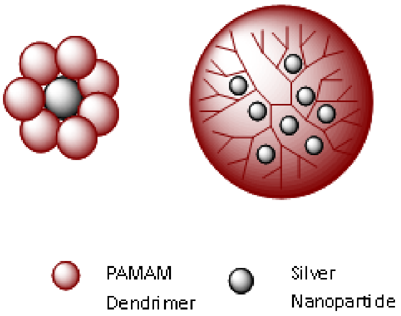

Figure 3: Schematic represent of two types of silver nanoparticle formation in PAMAM dendrimer. Left: Dendrimer Stabilized Nanoparticles (DSNPs); right: Dendrimer Encapsulated Nanoparticles (DENPs)

3.3. Fluorescence Spectroscopy. Figure 4 presents the fluorescence spectrum of the colloidal silver nanoparticles composite with PAMAM G 2.0. The fluorescence excitation at 410nm resulted in an emission peak at $\approx$ 510nm. When the concentration of silver increased from 200ppm to 800ppm the intensity of the fluorescence spectra was quenched. Collisional quenching occurred when the excited fluorophore made contact with an atom or molecule that could facilitate non-radiative transition to the ground state. In this case, the fluorophore was the primary amine and the secondary amine of PAMAM G 2.0

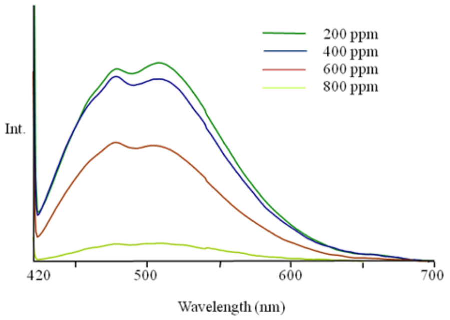

Figure 4: Fluorescence spectrum of colloidal Ag-PAMAM G 2.0

3.4. Antibacterial Activity Study. The bactericidal activity of Silver-PAMAM-Dendrimers composites against *Escherichia coli*, *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa* indicated the occurrence of larger zones of inhibition in case of Silver-PAMAM-Dendrimers mixture of 600ppm. At this concentration, the Silver-PAMAM-Dendrimers compounds were effective against both gram negative and gram positive bacteria. The results showed that *E. coli* and *P. aeruginosa* were the most resistant at concentrations below 200ppm (Figure 5).

Generally, the differences between the structure of the cell walls of gram-positive and gram-negative bacteria could influence how PAMAM dendrimers affected each organisms. PAMAM dendrimers were the most effective against gram positive organisms [14, 15]. Previous studies have shown that, the PAMAM dendrimers form small holes on the layer lipids of bacterial cells. These holes have the potential to remove lipid molecules from the surface. However, the cells recovered when the concentration levels of the PAMAM dendrimer decreased, are indicating that the damage was not permanent [16].

Experimental evidence suggests that even at high concentrations silver nanoparticles in liquid mediums, have limited application as biocide materials due to the fact that delays in the growth of bacteria is caused only by their low colloidal stability [17-19]. Possessing stable colloidal properties is an important factor for increasing durable antimicrobial activity. The silver-PAMAM-dendrimer synthesized in this study, exhibited stable colloidal properties for more than a year.

The biocidal activity of silver-PAMAM-dendrimer involves several steps (Figure 6). First, materials are absorbed by the cell surface and diffused through the cell wall. The adsorption process is electrostatic in nature and the cationic functionality of the amine-terminated PAMAM-dendrimers interacts with negatively charged constituents on the cell surface. The bacterial membrane maintains at biological pH due to the dissociation of excess carboxylic and other groups [20]. The membrane of bacterial cells is known to contain sulfur-containing proteins; these are preferential sites for silver NPs. According to the theory of hard and soft acids and bases, silver has an affinity for sulphur and phosphorus compounds [21]. Silver NPs migrate along with PAMAM dendrimer into the cell and react with other sulfur-containing proteins and phosphorus-containing compounds such as deoxyribonucleic acid (DNA) found inside the cell. When DNA molecules are in a relaxed state, the replication of DNA can be effectively conducted. When silver ions penetrate a cell, DNA is condensed and loses its ability to replicate leading to the cell death [22].

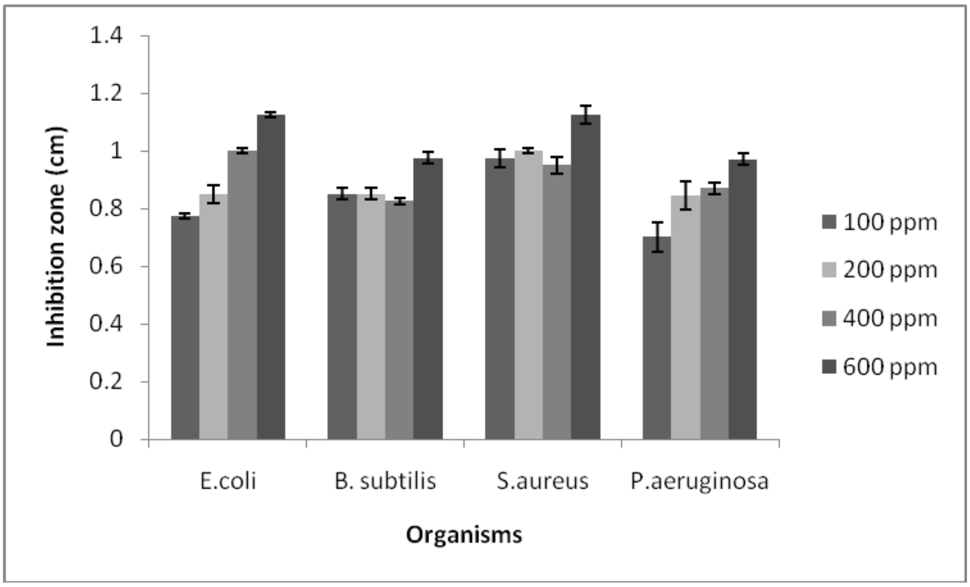

Figure 5: Bacterial growth inhibition zones induced by different PAMAM dendrimers concentrations

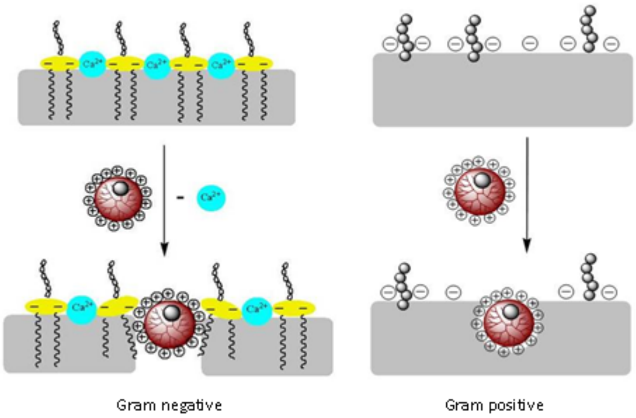

Figure 6: The suggested mechanism by which DSNPs penetrate into the cell wall of the gram positive and gram negative bacteria

Inside the cell, silver nanoparticles are released through a three stages process. First, water is diffused into the composite specimen. Secondly, the silver and water molecules react and lead to the formation of silver ions. Lastly, these silver ions are released through the compound to the aqueous environment causing the cytoplasm to leak leading to bacterial cell death.

4. CONCLUSIONS

In this study we have demonstrated that the synthesis of Generation 0.5 to Generation 4.0 poly (amidoamine) (PAMAM) dendrimers combined with silver in Generations 2.0, 3.0 and 4.0 in a colloidal solution is a simple method for creating novel antimicrobial agents. The silver-PAMAM-dendrimer used in this study exhibited bactericidal activities *in vitro*. The significant inhibition zones obtained at 600ppm revealed that silver-PAMAM-dendrimer could have a great potential to be used as an antiseptic material, which are in high demand for biomedical applications.

5. ACKNOWLEDGMENT

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