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Synthesis and Evaluation of Anovelantibacterial Nanoconjugate: Silver-Anionic-Linear-Globular Dendrimer

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Abstract: The elevated altitudes of control possible over the dendrimers architectural design are their shape, size, surface functionality and their branching length/density. The bioactive agents may be condensed into the interior of the dendrimers or physically adsorbed/chemically attached onto the dendrimer surface. In this case, the high density of exo-presented surface groups enables attachment of targeting agents or functionality that may moderate the antibacterial function of dendrimers. It has been shown that Modified dendrimers act as nano-drugs against bacteria, viruses and tumors. In the present study, a novel dendrimer based silver nanoparticle was synthesized and evaluated on different bacteria speceis as well. The results showed a very good comparavarable significant antibacteial effects for the proposed nanoconjugate. This paper demonstrates the potential of this new fourth major class of polymer architecture and indeed substantiates the high hopes for the future of dendrimers.

Key words: Dendrimer · Silver · Antibacterial Effects

INTRODUCTION

Conjugates of polymer-drugwere first proposed in the 1970s by Ringsdorf [1]. The molecular nanostructures development with well-defined particle shape and size is of prominent interest in biomedical applications with nanoconjugate. Constructs operates as carriers in drug delivery in the uniform size and nanometer in range to improve their ability to cross cell membranes and reduce the possibility of undesired clearance from the body through the spleen or liver.

Dendrimers are a novel class of polymers first reported by Vögtle and co-workers. They are kinds of polymeric architectures with diverse surface and chemical-related properties. Compared with traditional polymers, dendrimers have been claimed to be more

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prominent due to their characterizations such as: narrow polydispersity index, nanoscale (nanometersized polymers) architecture and large number of surface functional /reactive sites with the ability for bond formation with therapeutics [1].

These polymers are provided by using a divergent/convergent method, unique branching synthetic methodology, which result in the construction of materials with controllable shape, molecular weight, size (nm) and at times symmetric structures [2].The compact globular shape, size monodispersity and controllable 'surface' functionalities structure of dendrimers make them one of the best candidates for evaluation as drug carriers [3]. physically drug molecules inside the dendritic structure covalently attached drug molecules onto their surface.

Citric acid–polyethylene glycol–citric acid (CPEGC) triblockdendrimers biocompatible compounds containing G1, G2 and G3 were reported firstly by Namazi and Adeli [5]. Moreover, Alavidjeh and coworkers indicated that a biocompatible range for these dendrimers up to the concentration of 0.5 mg/ml and there were not observed any in vitro immunogenicity, cytotoxicity and hemotoxicity up to the related concentration in comparison with other denderimers [2-7].

Silver usage as an antimicrobial material has been known for a long time. Silver as an antimicrobial agent isefficient with low toxicity, which is especially significant in the topical antibacterial treatment of burn wounds, where transient bacteremia is commonly cited [4].

Silver complexes of dendrimers have antibacterial activity against *Escherichia coli* and it is enhanced by its combination with amoxicillin[10].

Easily alterable chemical agents of Silver with groups such as carboxylate ions on the dendrimer surfaces, so result in to form more stable bonds (due to greater thermodynamic stability, steric effects and ring stability of carboxylate ions versus the nitrate ions) with silver and prevent undesirable reactions with thiol, chloride and sulfate groups resulting from silver's poor water-stability prior to reaching their sites of actions.. (Silver nanoparticles encapsulated in glycogen biopolymer: Morphology, optical and antimicrobial properties-).Increasing the stability of these bonds wouldprevent from this undesirable reaction and systemic toxicities that are associated with silver therapy. Our purpose for studying these conjugates of dendrimer-Ag was to investigate the *in vitro* antibacterial activity of dendrimer-Ag conjugate and determine its MIC against *E.coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, then compared with $AgNO_3$.

MATERIALS AND METHODS

According to Namazi and Adeli [5]divergent methodthe G1 (MW: 1,000 Da) and the G2 (MW: 2,000 Da) were prepared.The silver nitrate($AgNO_3$), DCC and Citric Acid were purchased from Sigma-Aldrich, (Sigma-Aldrich Inc, St. Louis, MO, USA). Dialysisbag (molecular weight cut-off, 100–500 Da and 500-1000 Da) was obtained from Spectrumlabs (Spectra/por®, Rancho Dominguez, CA,USA). Three bacteria species including; *E.coli, S.aureus* and *P.aeruginosa*were donated by Pasteur institute (Tehran, Iran).

Statistical Analysis: All the experiments were performed in triplicate and the outcomes were compared using one-way ANOVA in order to mean comparison of more than two groups with SPSS software. Results were reported as meaningful for $P<0.05$.

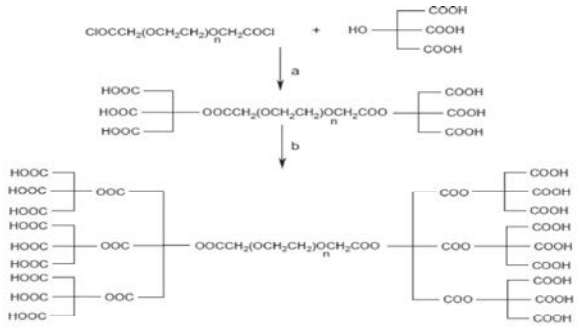
Synthesis: G1and G2 generations of the dendrimers were prepared and applied in the experiments. In brief, these dendrimers synthesized in two steps including esterification and combinationwith citric acid. The monomer units of ester-liked fragment were citric acid and diacyl halide poly(ethylene glycol) was used as the dendrimer core. The final complexis CA-PEG-CA triblock copolymer.

Diacid poly ethylene glycol was chlorinated using thionyl chloride (SOCl2) and reacted with citric acid as the monomer to generate the G1 (a). Then, theG1 was coupled to citric acid to produce the G2 with theaid of dicyclohexylcarbodiimide (DCC) in the pyridinemedium (b).

We obtained Compound G1 through the DCC–PEG–DCC reaction with citric acid. Then, the G2 was produced by coupling the G1 to citric acid.The reaction should be functionalized in dry condition. Then we used dimethyl sulfoxide (DMSO) as resolvent that reacted with $CaCl_2$ in portion of 10:1 was prepared to getdried condition (Scheme 1).

Preparation of the Silver Derivative: The dendrimers with carboxylate end groups were reacted with $AgNO_3$ in PBS. Typically 27.2 mg of $AgNO_3$ and 4 mg dendrimer (G2 generation) were dissolved in 5 ml of PBS, pH=7.2

Each dendrimer solution was then partially neutralized using 0.01 M NaOH solution, to provide the nucleophilic carboxylate end groups and Ag_2O.The dendrimer solutions, was added dropwise to the $AgNO_3$ solution with constant mild stirring in 24 hours period of time in dark situation. After combination of the yellow



Scheme 1: Dendrimer synthesis pathway

colored dendrimer with $AgNO_3$, it changed to the grey color.After 24 h, the solution passed through a filter paper, the brown colored Ag/dendrimer conjugate passed the filter and was accumulated in the container left additional Ag,which appeared as a dark spots on the filter paper.

Determination of Ag Concentration with Onductively Coupled Plasma (ICP) Mass Technique: In order to precise determination of Ag concentration we examined 1ml of conjugate with ICP-mass technique (IRIS intrepid-II XPS, Thermo Scientific, USA). The examination accuracy is in range of ppm (parts per million) to ppb (parts per billion).

Calculating and Optimizing of Incorporation Content (IC) and (Loading Efficieny) LE: After measurement of Ag concentration in order to determine the remained Ag in the dendrimer construction and its optimization in different conditions we investigated the data according to the below formula.

$$\text{IC:}\left(\text{wt\%}\right)=100\times\frac{\text{the total amount of Ag in carrier}}{\text{the amount of carrier added initally}}$$

$$\text{LE:}\left(\text{wt\%}\right)=100\times\frac{\text{the total amount of Ag in carrier}}{\text{the amount of carrier added initally}}$$

Fourier Transform Infrared (FT-IR) Assay: Conjugate structure were approved by (FT-IR) spectroscopy (14) (Thermo- Nicolet, NEXUS 870, Waltham, MA, USA) technique and spectral changes of the both generation of dendrimers were examined and compared with range of Ag/dendrimer conjugate. In the course of changes in the wave numbers of the functional groups in the IR spectra, we were able to illustrate the surface conjugation and elucidate which groups were related to the conjugation process. Ten milligram of each dendrimer generations and conjugate with sufficient amount of potassium iodide were mixed. Then, the pills were provided by using pill press machine. After that the pill was placed within the related machine. At the end the spectrum of the sample was detected.

Comparison of the Zeta Potential and Size Distributions (14): Both of the conjugate and dendrimers (0.1 mg/mL) was checked for any changes in the zeta potential and size distribution before and after the reaction with the $AgNO_3$ by DLS technique (Malvern, Zetasizer Nano ZS, Worcestershire, UK) in D.D.W.The measurement of molecular weight, zeta potential and measurement range are respectively, 10 to 2×10^7 Daltons, -120mVto +120mV and 0.6 m to6 nm.

AFM Assay (10-12): The resulting images of conjugate from AFM (DME, Denmark) compared with nanodendrimer images (G2 and G2-Ag).

Antibacterial Characteristics of Conjugate: After synthesis of appropriate conjugate and determination of its features, antibacterial characteristics must be specified. Thus, in order to determine the antibacterial properties, we should prepare the same concentration of Ag in two way of conjugate and $AgNO_3$, then compare with each other.After performing ICP-MASS method the Ag exact concentration in conjugate form was reported 0.2005 mg/ml. Because of 63.4 percent weight of Ag in $AgNO_3$ molecule, we should use 31.7 µg of $AgNO_3$, to obtain 0.2 mg/ml concentration of this molecule. In this study we preferred the well diffusion method to investigate the antibacterial effects. In addition, each well has a volume capacity of 100 µl of antibacterial solution. Therefore, 0.2, 0.1, 0.05 and 0.02concentrationsof Ag in two way of conjugate and $AgNO_3$prepared and the G2 dendrimer same concentration as a negative control was considered.The three strains of bacteria,-*E.coli*, *S. aureus* and *P.aeruginosa*were grown on MHA medium by surface culture method that each plate has four well containing four different concentration of antibacterial agents candidate such as dendrimer, $AgNO_3$ and conjugate separately. The plates were incubated at 37°C for 18 hours and then zone of inhibition was measured in laboratory and compared with each other. The minimum inhibitory concentration (MIC) of tested concentrations was determined at 37°C for 18 hours incubation.

RESULTS

Synthesis of Nanodendrimers: The G1 and G2nanodendrimerswere synthesized in the yield of 90% and 70% respectively.

Synthesis of Nanoconjugate: The Ag/dendrimer conjugate was produced in alkaline condition after 24 shaking on stirrer while placed in dark room to preventtheAg oxidation. Alkaline conditions result in oxidation of carboxyl group and to provide nucleophiliccarboxylate end groups. The yellow colored dendrimer after conjugation with $AgNO_3$ changed to the grey color that it indicates the accuracy of normal conjugation. At the end after filtration assay it results in the accumulation of brown conjugate. ICP-mass technique result after examination of each sample revealed as 0.2005 mg/ml.By using IC and LE related formula and putting the data on each formula the result of calculation is according to below:

$$\mathrm{LE}(\%) = \frac{0.2005 mg/ml}{\frac{27.2}{5} mg/ml} \times 100 = 3.686$$

$$\mathrm{IC}(\%) = \frac{200.5 mg/ml}{\frac{4000}{5} mg/ml} \times 100 = 25.0625$$

FT-IR: Considering that hydroxyl and aliphatic peaks (citric acid carbonyl, aliphatic carbon of citric acid and esteric bond) in FT-IR of G2 have a more distinct strength than of G1. Therefore, we can conclude that the mentioned peaks confirm the two generation of dendrimer (considering functional group and both weaknesses and intensities of additional group peak) (Figure 1).

FT-IR results of the conjugate products proved distinct alterations in the spectra indicating the conjugation at the dendrimer surfaces via carboxylate groups. For example, the obvious increase in the wavelength may have resulted from the reaction of oxygen (as a strong base) of the carboxylate (–COO–) ion with Ag(as a weak Lewis acid).This type of bond polarizes, deshields the carbon atom of the –COO– group, this induces a small negative charge on it and finally increases the possibility of the carbonyl group having a greater strength and wave number. Moreover, the appearance a doublet bands near the G2?Ag conjugates in comparison with the G2 dendrimer that is related to the stretching of the groups.

Size and Zeta Potential Distribution: The G2 dendrimer has a more negative charge and size in comparison with G1 dendrimer that is due to much more carbonyl residue in dendrimer surface (Table 1). After the conjugatewas formed from the dendrimer, as we expected, negative chargeincrement around 30 mv in the zeta potential indicating that Ag_2O interacted with dendrimerdue to Ag affinity to attract the oxygen atoms in the carbonyl groups. In addition35nmincrements in the size/poly dispersity were seen in the conjugates contrasted with the dendrimer that shows Ag_2O was interacted with both carbonyl groups and it loaded in dendrimer construction and it changes the dendrimer conformation. The 35nm size increment of dendrimer is not alone due to interaction of Ag_2O with carbonyl groups but also loading in dendrimer*structure. The 12nm peak caused by spontaneous reaction of Ag derivatives is not related to conjugate (Figures 2-3).

Table 1: Size and zeta potential distributions of the dendrimers and conjugateafter reaction with$AgNO_3$

Types	Size distribution(nm)	Zeta potential (mv)
G1	70	-1.5
G2	91.5	-3.38
Conjugate (G2+$AgNO_3$)	126.8	-33.7

Table 2: The antibacterial effects of AgNO3 and conjogate. Mean ± SD (n = 3) of the data are depicted in each cell.

Concentration of Ag (mg/ml)		0.2	0.1	0.05	0.02
E.coli	In $AgNO_3$	18.33±0.57			
	16.33±0.57	13	10.66±0.57		
	In Conjugate	25.33±0.57	18±1	14.33±0.57	Negligible (about 10)
S.aureus	In $AgNO_3$	17.33±1.15	15±1	10.66±0.57	Negligible
	(about 10)				
	In Conjugate	31.66±1.52	24.66±0.57	22.33±0.57	12.66±0.57
P.aeruginosa	In $AgNO_3$	18.66±0.57	17±1	14.66±0.57	11.66±0.57
	In Conjugate	24.33±0.57	17±1	13.33±0.57	Negligible (about 10)

Table 3: The minimum inhibitory concentrations of AgNO3 and conjogate. Mean ± SD (n = 3) of the data are depicted in each cell.

	MIC (mg/ml)	
E.coli	$AgNO_3$	0.05
	Conjugate	0.05
S.aureus	$AgNO_3$	0.05
	Conjugate	0.02
P.aeruginosa	$AgNO_3$	0.02
	Conjugate	0.05

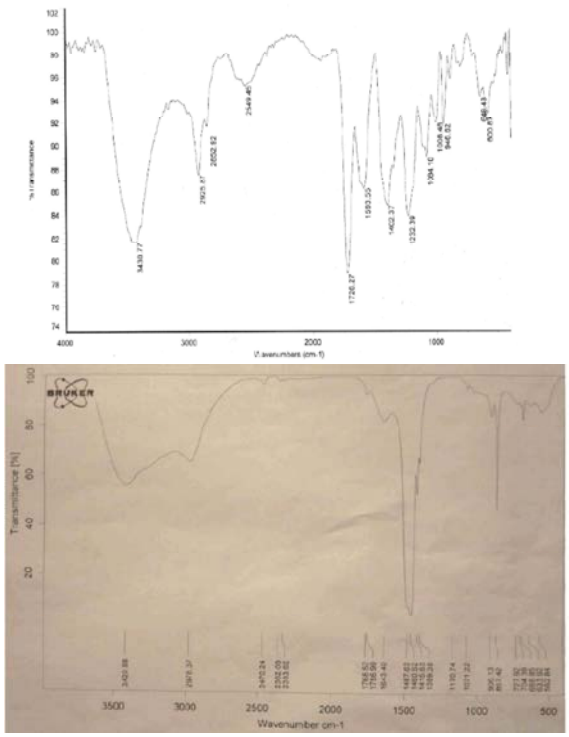


Fig. 1: FT-IR spectra of the dendrimers: (a) G2, (b) G2-Ag

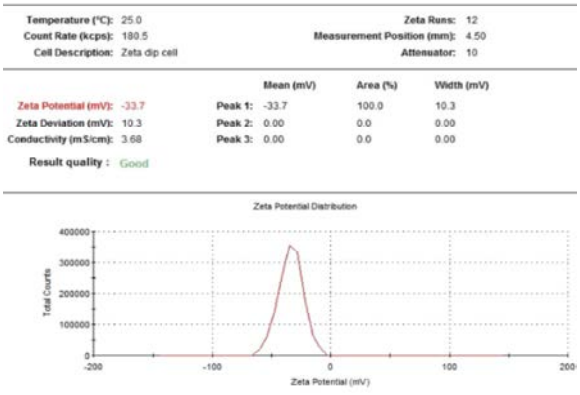


Fig. 2a: Conjugate zeta potential

AFM Results: According to data the AFM images of the dendrimer and conjugate was taken and compared with each other (Figure 4).

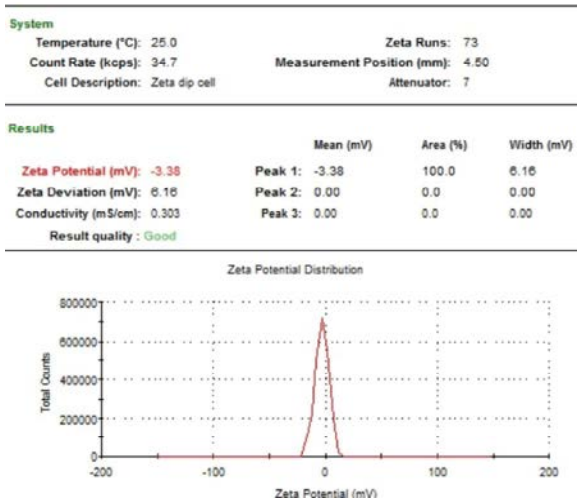


Fig. 2b: G2dendrimer zeta potential

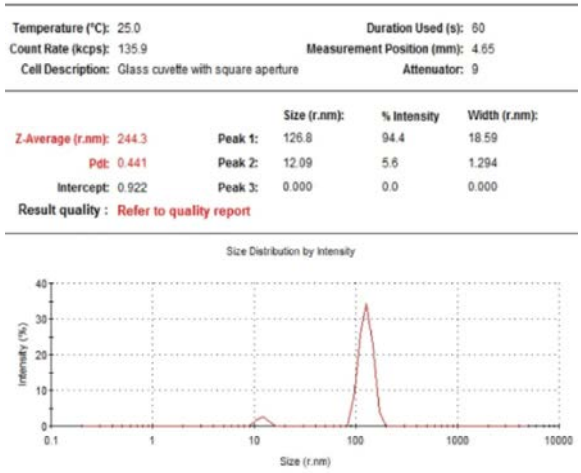


Fig. 3a: Conjugate size

Antibacterial Results: Each of four concentration effect of Ag in two ways of $AgNO_3$and conjugate was threated against bacteria. The results are shown in the Table (2). In addition, the results indicate that the pure dendrimer just has a function as a carrier and of no antibacterial effect.

MIC Results: The minimum inhibitory concentrations (MIC) for both solutions are shown in Table (3).

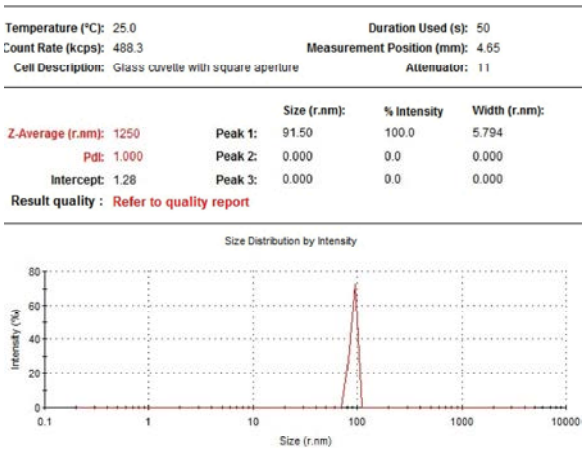


Fig. 3b: G2 dendrimer size

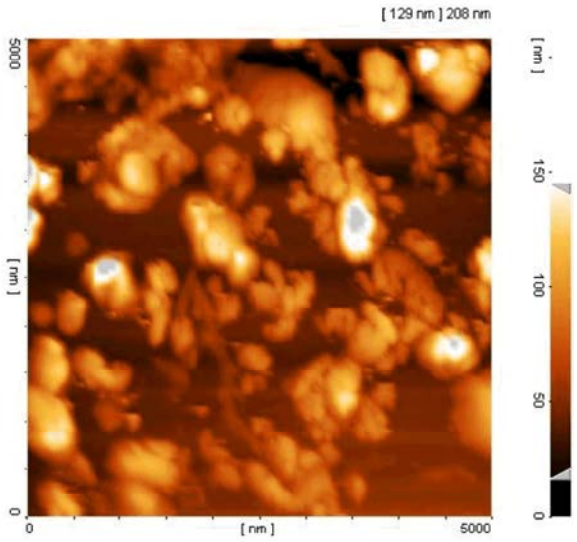


Fig. 4: AFM images of the nanosized conjugate

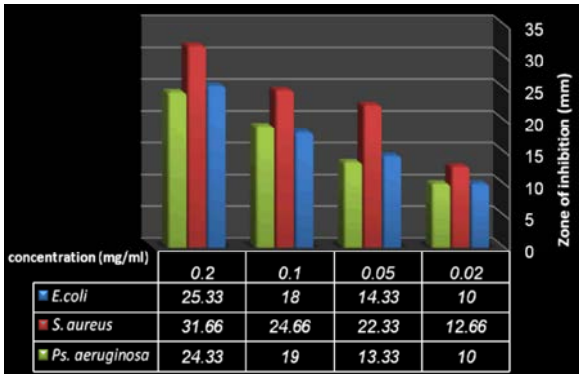


Fig. 5: Antibacterial effect of conjugate. Mean ± SD (n = 3) of the data are depicted in each bar, the two connector lines show the two groups with statistical significance (post-hoc test) P<0.05 (significant).

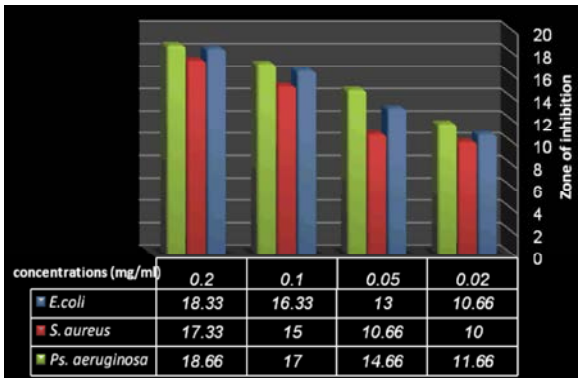


Fig. 6: Antibacterialeffect of AgNO3.Mean ± SD (n = 3) of the data are depicted in each bar, the two connector lines show the two groups with statistical significance (post-hoc test) P<0.05 (significant).

DISCUSSION

Several approaches with silver and with diverse organisms have shown that silver is an efficient antimicrobial agent against several microorganisms-(28-30-).Manipulating these agents with dendrimers provide more effective biocidal activities because a wide range number of antibacterial agents can be provided by dendrimers[10].Some bioactive materials, such as metal ions, metals and organic molecules, consequently, may be combined in variable concentrations within one nanoscopic delivery vehicle when dendrimers are used [11].

Ghosh and colleagues have shown that Silver–dendrimer complexes were prepared with $AgNO_3$is more effective than other form of Ag. We used three types of bacteria to assess antibacterial activities of desired conjugate and $AgNO_3$.According to our results generally the generated conjugate has a more efficient antibacterial activity in comparison with $AgNO_3$beacause of the proving reasons such as: first the silver not only exists in cell wall and membrane of bacteria but also presents within the bacteria and our results demonstrating that there is no antibacterial activity of unconjugated dendrimer thus it acts as a transmembrane carrier second the mor enegative charge of the conjugate results in an increased number of sites being able to interact with the positively charged componentsof the bacteria surface in comparison with the $AgNO_3$ third in this study due to large number of negative charged terminal groups (18 carboxylate groups around a 91 nm dendrimer) are accumulated within a relatively thin shell of anionic linear-

globular dendrimers, very high local concentration of silver could be provided by interaction of silver with carboxylate groups. Last reports indicated that the use of antimicrobial polymers has a higher efficiency compared with existing antimicrobial agents. Because of dendrimer unique structures, usage of them could be beneficial as dendrimers are able to solubilize various organic materials that are usually considered insoluble in water through guest-host interactions [12].

Antimicrobial agents are mainly made up of small molecules and these molecules with dendrimers have been used to as polyvalent biocides [13].

The silver-dendrimer conjugate appeared with antibacterial activity better than $AgNO_3$ solutions. Due to the secondary silver compounds similar limited solubility that was formed in the test media, all silver nitrate solutions exhibited approximately the same activity probably [14]. In the absence of dendrimers, silver ions precipitate in the form of insoluble silver salts after contacted with chloride and sulfate ion containing solutions. When conjugated to a dendrimer, the silver ions were transformed into constant silver nanococonjugate that remained soluble in the solvent.

Because the dendrimer host solubility, make it possible to deliver the immobilized silver in the agar medium by its own diffusion. The silver cluster that has extremely high surface area remains active. The generated conjugate was act as antibacterial agent against *S. aureus*, *P. aeruginosa* and *E. coli*. The trapped silver compounds and silver appeared high antibacterial activity in several cases without the reduction in solubility.

Based on the current obtained results it is suggested to further investigate the clinical aspects of such nanosized conjugated of silver in the near future.

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