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Synthesis of the erythromycin-conjugated nanodendrimer and its antibacterial activity

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ABSTRACT

The development and spread of bacterial resistance to antimicrobial drugs necessitates the need to search for novel and effective antimicrobial agents. In the last few decades, innovative nanomaterials are attracting increasing attention and, among them, dendrimers have shown wide application in the various fields. In the current study, the two generations of an anionic linear- spherical nanodendrimer G1 and G2 were synthetized and compound G2 of nanodendrimer conjugated with erythromycin. The structures of the nanodendrimers were characterized by FTIR spectroscopy, zetasizer, and scanning electron microscopy (SEM). The antibacterial activity of the erythromycin-conjugated nanodendrimer and erythromycin alone were evaluated by the microdilution method against *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, and *Pseudomonas aeruginosa*. The size of first and second generation of nanodendrimer, and the erythromycin-conjugated nanodendrimer was 75, 95, and 65.6 nm, respectively. The drug loading percentage of the nanodendrimer conjugates was obtained to be in 35.2%. In our study, the erythromycin-conjugated nanodendrimer showed significantly more bacteriostatic and bactericidal activities against all four studied bacteria than erythromycin alone. Our study's results highlight that the erythromycin-conjugated nanodendrimer is a highly effective agent against Gram positive and negative bacteria. The antibacterial properties of erythromycin combined with the targeting potential of the nanodendrimer can lead to sustained intracellular delivery of therapeutic agent.

1. Introduction

Bacteria are common infective agents producing a wide variety of diseases. Today, treatment of bacterial infections due to the emergence of new infectious agents and increasing the number of drug-resistant pathogens has been still stayed as an important problem. The emergence of resistant bacterial pathogens occurred in other times by the intensive and incorrect use of antimicrobial agents (Ghosh et al., 2018; Tabatabaei et al., 2017; Tanwar et al., 2014). In recent years, many studies have focused on developing novel and effective antimicrobial agents. Additionally, the goal of successful medical treatment is delivery of a proper concentration of drug to the proper location so as to achieve the most effective therapeutic and lowest toxicity (Chatterjee et al., 2008; Moritz and Geszke-Moritz, 2013).

Most recently using the new technologies of human knowledge has been significantly considered in the medical and medicinal fields. Meanwhile, nano-technology has opened a new era in front of researchers' eyes. The field of nanomedicine is emerging as nanosystems enable scientists to enhance more favorable pharmacodynamics and pharmacokinetic properties and can even be used in combination with traditional drugs (Authimoolam and Dziubla, 2016; Biswas et al., 2014). The dendrimers are one of the most important nanostructures investigated in the field of nanomedicine that have served as a unique platform for drug delivery. These nanomaterials are made of a central core, derived units as branches, and superficial groups (Abbasi et al., 2014; Jain and Asthana, 2007). The unique characteristics of dendrimer structure are its heavy-branched and regular structure, terminal multiactive groups and the empty cavities between the branches. These empty spaces have ability of accepting the guest molecules in various sizes (Charles et al., 2012; Strydom et al., 2013).

Erythromycin is used as a macrolide in treatment of many bacterial infections. Unfortunately, the emergence of resistance to these antibiotics in recent years has become an increasing health concern worldwide. The use of nanodendrimer in combination with traditional

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antimicrobial agents such as erythromycin can increase its therapeutic effects. Furthermore, conjugation of poorly water-soluble drugs to the soluble nanodendrimer can increase the bioavailability as well as drug solubility (Bosnjakovic et al., 2011; Jelić and Antolović, 2016; Tulu et al., 2009).

Given the importance of using nanoparticles in the treatment of infectious diseases, the need for study is more than ever. Therefore, the aim of this study was to synthesis of the erythromycin-conjugated nanodendrimer and evaluation of its antibacterial activity.

2. Materials and methods

2.1. Material and bacterial isolates

Poly(ethylene glycol) methyl ether (m-PEG), dicyclohexylcarbodiimide (DCC), ethyl dimethylaminopropyl carboxamide (EDC), dimethyl sulfoxide (DMSO) and erythromycin were obtained from Sigma (Sigma-Aldrich, MO, USA). Four standard strains of bacteria were purchased from Pasteur Institute (Tehran, Iran).

2.2. Synthesis of the first (G1) and second generation (G2) nanodendrimer

In the present study, the anionic linear-globular nanodendrimers G1 and G2 (having PEG core and citric acid periphery surface) were synthetized according to Namazi and et al. method with a few modification (Namazi and Adeli, 2003).

Synthesis of compound G1 of nanodendrimer was initiated through adding 2 mL of PEG-600 as a dendrimer core to the Dried DMSO. DMSO worked as a basis of the reaction. Then 1.8 g of DCC was added and mixed for 5 min in the condition of darkness and vacuum. DCC activated PEG by substitution with hydrogen atom of carboxylic acid groups. Finally, 1.7 g citric acid was added to the solution and within 30 min, the color of the tick solution changed to orange with stench. Reaction was terminated with adding water and after filtration; yellow-brown solution of nanodendrimer was obtained. The product of G1 was separated and purified by dialysis. Pore size of dialysis bag was 500 Da. Since the presence of impurity could interact with the next step of synthesis, so the column chromatography used as a purification method. Then the samples were lyophilized for DMSO removal.

To synthesize nanodendrimer G2, 500 mg DCC was added to 310 mg solution of nanodendrimer G1 in DMSO and stirred for at least 15 min and thereafter yellow-brown color was appeared. In the next stage, 1 g citric acid was added and after 30 min, 500 mg DCC was repeatedly added to the solution and stirred for 4 days at room temperature. The change in the color of the solution to dark cream verified the synthesis of the G2 nanodendrimer. As synthesis of the nanodendrimer, reaction was terminated with water addition, solution was filtrated, dialyzed (pore size of 1000 Da), passed through the column chromatography process, and powder was lyophilized.

2.2.1. Confidence of G1 and G2 synthesis

Fourier Transform Infrared (FTIR) analysis was carried out to confirm the structure of G1 and G2 nanodendrimers. Size and electric charge of the nanodendrimers was determined by zetasizer (dynamic light scattering, DLS).

2.3. Preparation of erythromycin-nanodendrimer conjugates

Tendency of erythromycin to the activated carboxyl groups of nanodendrimer leads to the reaction and incorporation of erythromycin into the nanodendrimers. First the G2 lyophilized dendrimer and DCC or EDC were stirred for at least 15 min to activate functional groups of G2 to make it ready for conjugation with erythromycin. After that, 500 mg erythromycin was added to activate nanodendrimer and was stirred for 7 days. Chromatography lyophilization was used as the previous step to yield purity.

2.3.1. Confidence of erythromycin conjugation

FTIR and scanning electron microscopy (SEM) analysis was carried out to confirm the structure and morphology of erythromycin-nanodendrimer conjugates, and zetasizer was used to determine the charge and size.

2.3.2. Determination of drug loading

Drug loading was determined following the indirect method (free drug concentration in supernatant). Percentage of drug loading was evaluated by determining the amount of free erythromycin in the erythromycin-nanodendrimer conjugate solution which was separated by using the cooling centrifuge (12,000 rpm for 20 min at 4 °C). The concentration of erythromycin in the supernatant was determined using UV–visible spectrophotometer at 280 nm. The drug loading were calculated by the following equations:

$$\text{Drug loading efficiency} = (\text{weight of loaded drug in nanodendrimer} / \text{weight of feeding drug}) \times 100\%.$$

2.4. Antibacterial activity assay

In the present study, the antibacterial activity of the erythromycin-conjugated nanodendrimer and erythromycin alone were evaluated against *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *S. saprophyticus* ATCC 15305, and *Pseudomonas aeruginosa* ATCC 27853. For this, two antimicrobial assays including the minimum inhibitory concentration (MIC) assay and the minimum bactericidal concentration (MBC) assay were applied.

2.4.1. Determination of the minimum inhibitory concentration (MIC)

The MICs were determined by the broth microdilution method and interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2004). Briefly, Mueller Hinton broth (Becton Dickinson, USA) containing two-fold concentration increments of antimicrobial agents were added to 96-well microdilution plates. Test organism suspension equal to a 0.5 McFarland standard was further diluted and added to the plates to achieve a final inoculum of 5×10^5 CFU/mL. The plates were incubated for 18–20 h at 37 °C in ambient air. Media containing bacteria alone was used as positive control. Noteworthy, this procedure was repeated three times. The MIC was defined as the lowest agent concentration at which observable growth was inhibited.

2.4.2. Determination of the minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was also assessed according to the CLSI guidelines (Wayne, 2004). In brief, after completion of the MIC assay, 10 µL from each clear well containing test compounds was subcultured to Mueller Hinton agar (Becton Dickinson, USA). The plates were incubated for 24 h at 37 °C in ambient air. The colony forming units (CFU) were determined visually. The MBC was taken as the concentration at which a three-log decrease in bacterial growth (> 99.9%) was detected compared to the initial inoculum. Experiments were performed in triplicate.

3. Results

3.1. Characterization of nanodendrimers

The sizes and zeta potential distributions of the nanodendrimers and erythromycin-conjugated nanodendrimer in concentration of 0.1 mg/ml were measured by DLS (Figs. 1 and 2). The G1 nanodendrimer size was obtained 326 nm for the first time but this size is unreal and so big and represents the aggregation of dendrimers together. After preparing a more diluted sample and 20 min of sonication, the particles size was determined 75 nm. However, this problem was resolved for G2

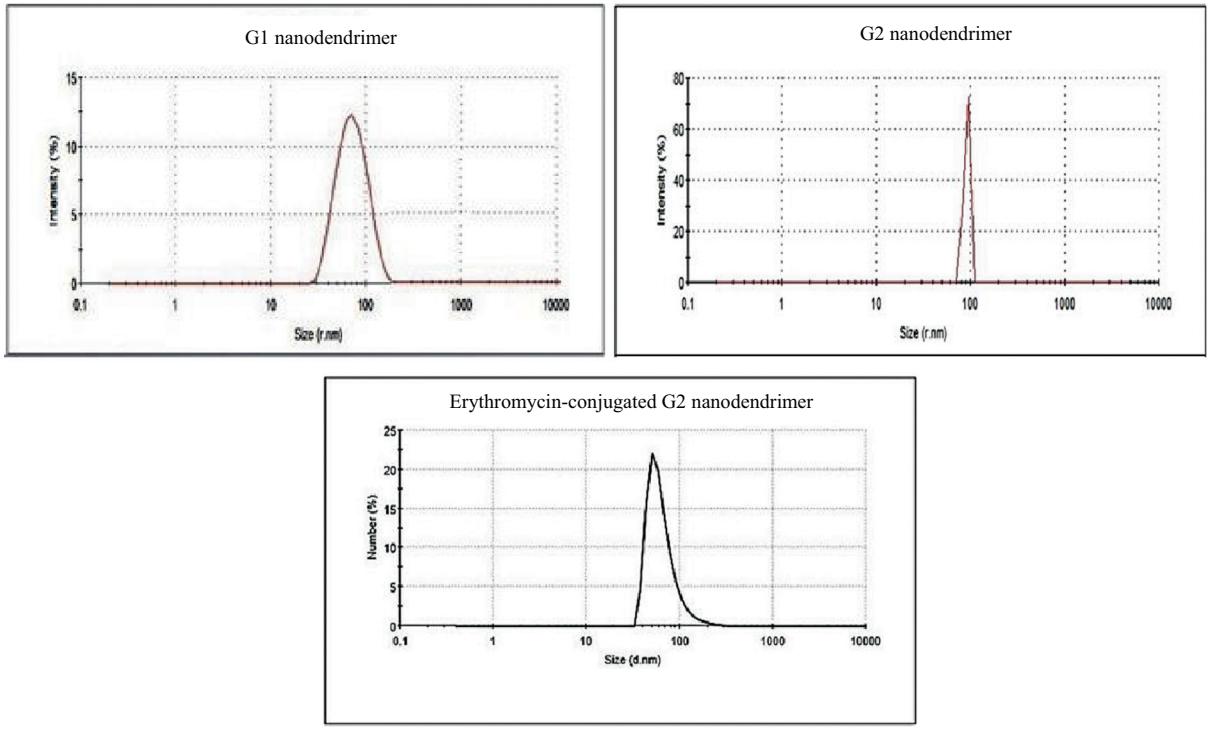

Fig. 1. Particle size of the nanodendrimers.

nanodendrimer and the particle size was about 91 nm. Furthermore, size of erythromycin-conjugated G2 nanodendrimer was obtained about 65.6 nm. It is noteworthy that the surface charge of nanodendrimer G1, G2 and the erythromycin-conjugated nanodendrimer was −5.7, −3.3 and +5.2 mV, respectively.

Moreover, the morphology and size of the erythromycin-conjugated nanodendrimer was characterized using SEM. The SEM image of erythromycin-conjugated nanodendrimer clearly showed the spherical dendrimer in different sizes (Fig. 3). Generally, the mean nanodendrimer size which was obtained by DLS was larger than that determined by SEM. This difference is due to aggregation of the nanodendrimer in aqueous solutions.

3.2. FTIR spectroscopy

FTIR spectroscopy was used to determine the structure of the

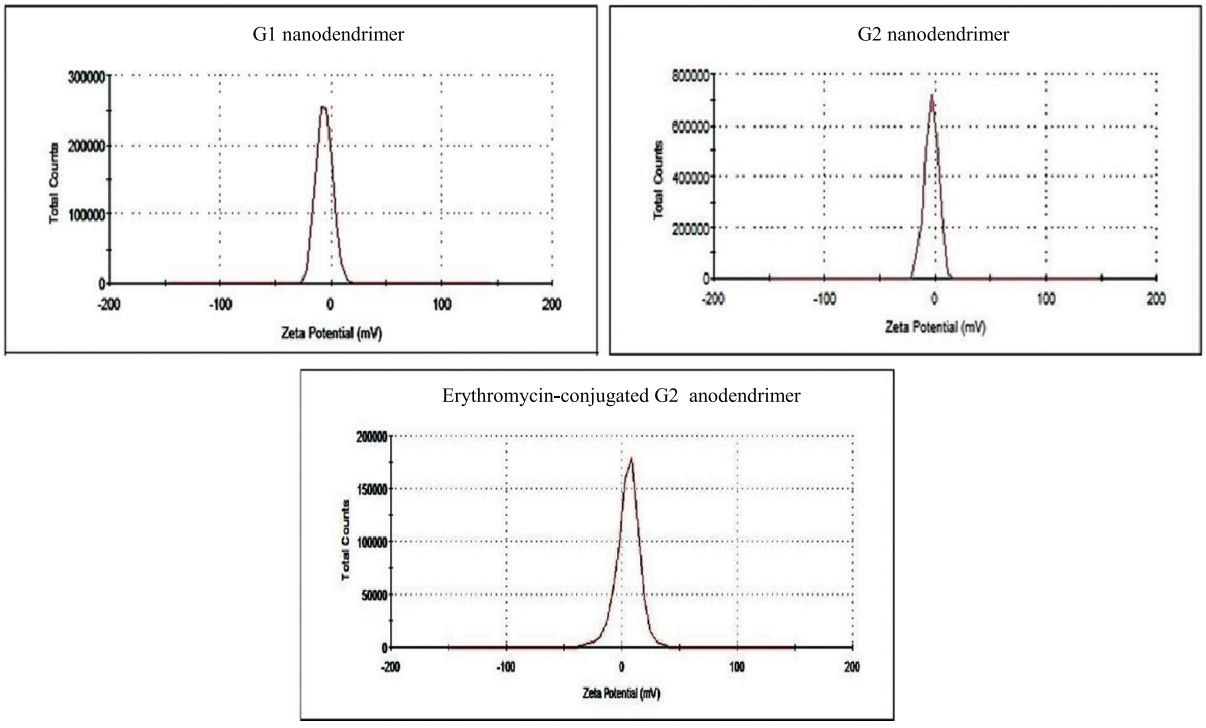

Fig. 2. Zeta potential of the nanodendrimers.

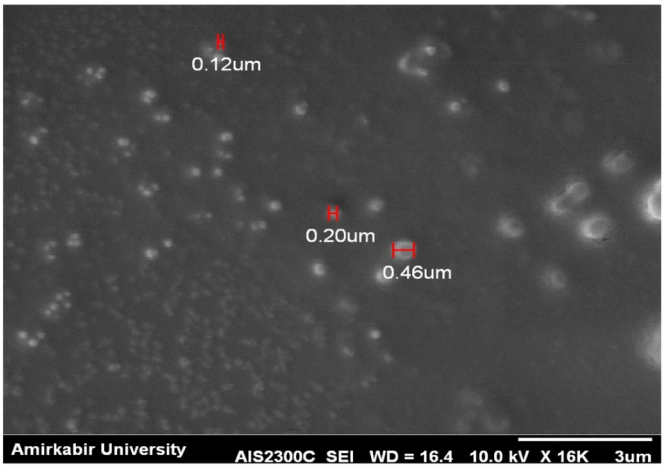

Fig. 3. SEM image of erythromycin-conjugated nanodendrimer.

synthetized products. FTIR spectrum of G1 nanodendrimer is shown in Fig. 4. The observed peak in 1279 cm^{-1} is related to C—O groups that confirmed the formation of the ester bond between PEG and citric acid, so represents the synthesis of G1 nanodendrimer. The 1154–1279 cm^{-1} range shows alcoholic C—O in PEG and citric acid. A broad peak at 3200–3500 cm^{-1} is related to the vibration of O—H bond.

In FTIR spectrum of G2 nanodendrimer (Fig. 4), the 1232 cm^{-1} peak shows C—O group that confirms the formation of the ester bond between terminal groups of G1 dendrimer and new citric acid groups, and G2 nanodendrimer synthesis. Also a broad peak observed at 2500–3430 cm^{-1} which is related to acidic OH in citric acid.

Finally, in FTIR spectrum of erythromycin-conjugated G2 nanodendrimer (Fig. 4), a broad peak observed at 1022–1196 cm^{-1} is related to C—O groups that confirmed the formation of the ester bond between nanodendrimer carboxyl groups and erythromycin, so represents the synthesis of erythromycin-conjugated nanodendrimer. Furthermore, the 1351 cm^{-1} peak is for C—O groups in the erythromycin. The broad peak at 3200–3405 cm^{-1} is assigned to the vibration bonds of OH in erythromycin. The peak around 1724 cm^{-1} attributed to C=O bond in the nanodendrimer.

3.3. Percentage drug loading of erythromycin-conjugated nanodendrimer

The drug loading percentage of the erythromycin-conjugated nanodendrimer was obtained to be in 35.2%.

3.4. Antibacterial activity assay

In this study, the in-vitro antibacterial activity of the erythromycin-conjugated nanodendrimer was evaluated against both Gram-negative (*P. aeruginosa*) and Gram-positive (*S. aureus, S. epidermidis,* and *S. saprophyticus*) bacteria. The MICs and MBCs erythromycin alone and the erythromycin-conjugated nanodendrimer against the bacteria studied are shown in Table 1. The results indicated that the erythromycin-conjugated nanodendrimer was 2- to 16-fold more active than erythromycin against the bacteria. The most antimicrobial activity of the nanodendrimer was observed against *S. saprophyticus*.

4. Discussion

In recent years nanometric polymeric carriers have been extensively used for drug and gene delivery. Meanwhile, dendrimers has gained

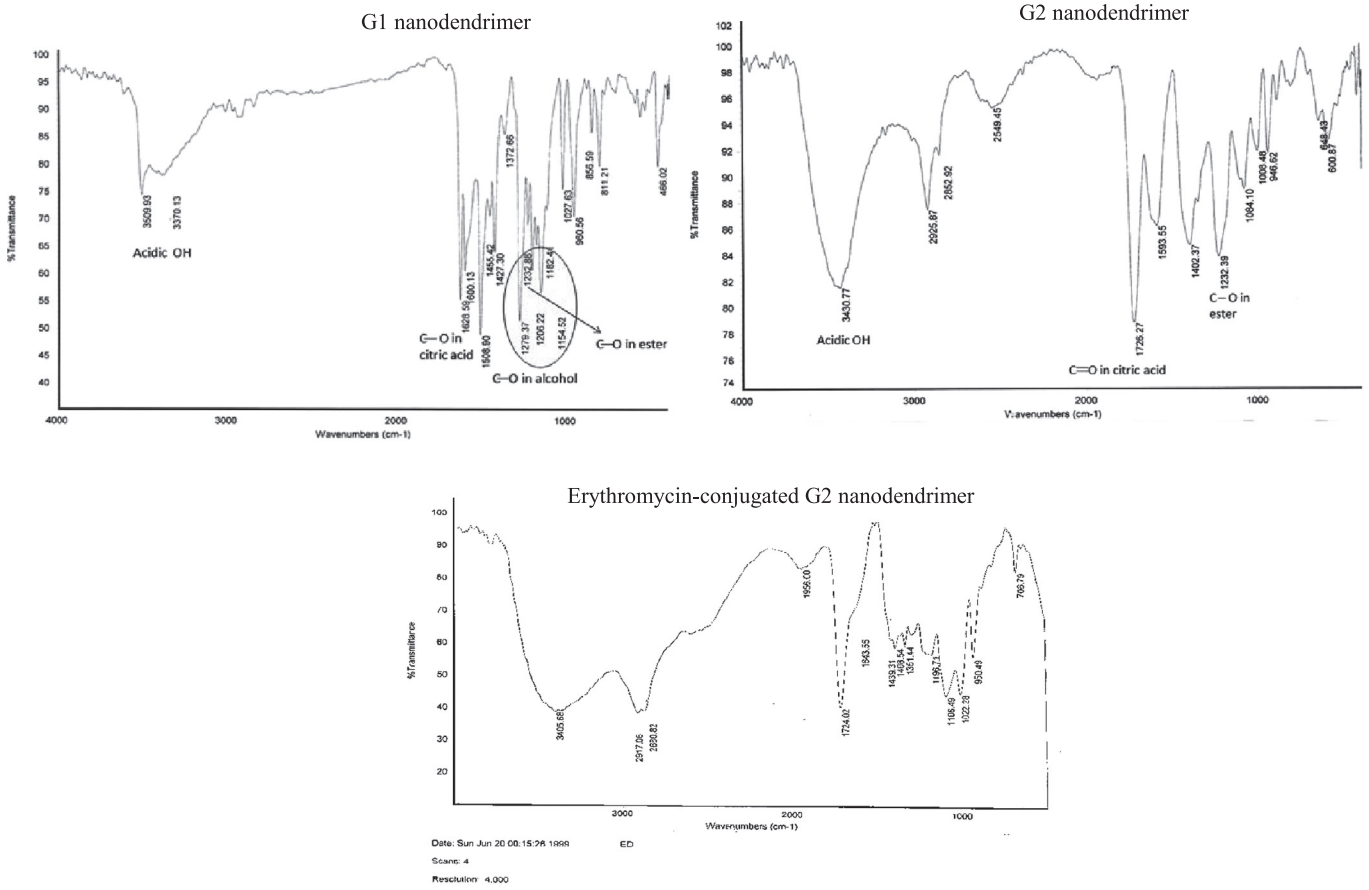

Fig. 4. FTIR spectra the nanodendrimers.

Table 1
MIC and MBC values of erythromycin and the erythromycin-conjugated nanodendrimer.

Bacteria	Erythromycin		Erythromycin-conjugated nanodendrimer	
	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)
P. aeruginosa ATCC 27853	128	256	32	64
S. aureus ATCC 29213	16	32	8	16
S. epidermidis ATCC 12228	128	256	16	16
S. saprophyticus ATCC 15305	0.25	1	0.125	0.25

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.

popularity in last two decades due to their stability, nanoscale size, multifunctionality, biodegradable nature and non-immunogenic characteristics. Furthermore, the unique structural feature of dendrimers is their regular branched structure, and the empty cavities between the branches that this space has ability of accepting guest molecules in different sizes (Bosnjakovic et al., 2011; Jain and Asthana, 2007; Strydom et al., 2013).

In the present study, the anionic linear-globular nanodendrimers containing citric acid and PEG as the core were synthetized according to Namazi and et al. method (Namazi and Adeli, 2003). The sizes of the nanodendrimers G1 and G2 were 75 and 91 nm, respectively. The synthesis of the nanodendrimers and the presence of functional groups could be confirmed using different methods including FTIR spectroscopy. FTIR analysis confirmed that the nanodendrimers G1 and G2 were synthesized, owing to typical peaks of C—O group such as 1279 and 1232 cm^{-1} which are attributed to the formation of the ester bond between PEG and citric acid and between terminal groups of G1 dendrimer and new citric acid groups. The nanodendrimers synthesized in this study are in the nano-scale and have not been reported any toxicity and immunological effects for them. Furthermore, these compounds are biocompatible. Because PEG which used as the core in the dendrimers and citric acid as the dendrimer branches are biocompatible, as well as citric acid is an intermediate compound in metabolic pathways (Akram, 2014; Twibanire and Grindley, 2014). It is noteworthy that in the dendrimer synthesis method in this study, not only used much less toxicity compounds but also reduced the reaction time. In the study, DCC was used instead of chlorinated compounds such as dichloromethane that are commonly used in the synthetic reactions as activator components. The chlorinated compounds are very toxic and can damage the human respiratory system (Henschler, 1994).

The dendrimer G2 synthesized in the present study is completely soluble in water, so it is a good candidate to increase solubility of the insoluble drugs in water. In the study, erythromycin was conjugated with the nanodendrimer G2. FTIR analysis confirmed that the erythromycin-conjugated nanodendrimer was synthesized, having typical peaks of C—O groups at 1022–1196 cm^{-1} range which are attributed to the formation of the ester bond between nanodendrimer carboxyl groups and erythromycin. Moreover, the nanodendrimer conjugate had a slightly positive zeta potential (5.2 mV) compered than the nanodendrimer G2 (− 3.3 mV), which is due to the bonding of the positively charged erythromycin to superficial carboxyl groups of the nanodendrimer.

Finally, in this study the antibacterial activity of the erythromycin-conjugated nanodendrimer was evaluated against Gram positive and -negative bacteria. The results indicated that the erythromycin-conjugated nanodendrimer was 2- to 16-fold more active than erythromycin against the bacteria. In addition to the role of the dendrimer in increasing the aqueous solubility of erythromycin, the antibacterial activity of the nanodendrimer conjugates can be due to the electrostatic interactions between positively charged the erythromycin-conjugated nanodendrimer and the negatively charged bacterial membrane that resulting in the increase of membrane permeability and bacterial lysis (da Silva Santos et al., 2016; Madaan et al., 2014). Noteworthy, it is the first time that these nanodendrimer are used for erythromycin delivery and showed significant effects in facilitation of delivering the drug such as erythromycin through the bacterial cell wall. Several studies have investigated the antibacterial activity of various dendrimers and drug-conjugated dendrimers. Lopez et al. studied the antibacterial activity of a series of amino-terminated poly(amidoamine) (PAMAM) dendrimers modified with PEG. The results showed that PEGylation of the dendrimers decreased their antibacterial activities. This reduction in antibacterial activity was likely due to the decrease in the number of protonated amino groups and shielding of the positive charges by the PEG chains, thus decreasing the electrostatic interactions of the dendrimers with the negatively-charged (Lopez et al., 2009). In another study, the use of PEGylated poly (propylene imine) (PPI) dendritic architecture for the delivery ciprofloxacin resistant bacterial strains was studied. The study showed that the ciprofloxacin loaded dendrimer has significant antibacterial activity than the plain PPI dendrimer (Karthikeyan et al., 2012). Contrary to our study, in the study of performed by Bosnjakovic and colleagues (Bosnjakovic et al., 2011), the antibacterial activity of the poly(amidoamine) dendrimer-erythromycin conjugates against *S. aureus* was comparable to free drug. They showed that this may be because the gram-positive *S. aureus* has a thick cell wall, and the neutral dendrimer may not provide a significant intracellular transport advantage to the free drug.

Overall, the present study indicated that the erythromycin-conjugated nanodendrimers as a biocompatible compound have a high drug payload, improve the solubility of the drug, and could lead to improved antibacterial activity. Our study results encourage further investigation of the potential of anionic drug-conjugated nanodendrimer as a new class of antibacterial agents that may be less likely to induce bacterial resistance than conventional antibiotics.

5. Conclusion

In this study, the erythromycin-conjugated nanodendrimers showed significantly more antibacterial activity than erythromycin against Gram positive and -negative bacteria. These nanodendrimers have a high drug payload and potential of passing through biologic membrane such as the bacterial cell wall. Thus linear-globular nanodendrimers could be a promising drug delivery system in the treatment of bacterial infection.

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