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Streptomycin-anionic linear globular dendrimer G2: Novel antibacterial and anticancer agent

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Abstract. Recent researches demonstrated well promising anticancer activities for antibiotics. Such effects would be significantly increased while nanoparticle based delivery systems were applied. In this study, the goal was aim to improve anticancer and antitoxic effects of Streptomycin by loading on special kind of dendrimer (anionic-linear-globular second generation). In the current study, Size and zeta potential as well as AFM techniques have been used to prove the fact that the loading was performed correctly. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the drug loaded on dendrimer nanoparticle were determined and compared with both of dendrimer alone and free drug with respect to staphylococcus aureus as the test microorganism. The anticancer activity among three groups including Streptomycin, Streptomycin –G2 dendrimer, and control was measured in vitro. In vitro studies showed that G2 anionic linear-globular polyethylene-glycol-based dendrimer, which loaded on Streptomycin was able to significantly improve the treatment efficacy over clinical Streptomycin alone with respect to proliferation assay. Maximal inhibitory concentration (IC50) was calculated to be 257 μg/mL for streptomycin alone and 55 μg/mL for Streptomycin –G2 dendrimer. In addition, Streptomycin –G2 dendrimer conjugate prevented the growth of MCF-7 cancerous cells in addition to enhance the number of apoptotic and necrotic cells as demonstrated by an annexin V-fluorescein isothiocyanate assay. Streptomycin –G2 dendrimer conjugate was able to increase Bcl-2/Bax ratio in a large scale compared with the control group and Streptomycin alone. Based on results a new drug formulation based nano-particulate was improved against S. aureus with sustained release and enhanced antibacterial activity as well as anticancer activity shown for functional cancer treatment with low side effects.

Keywords: antibacterial activity; anticancer; streptomycin; breast cancer; staphylococcus aureus

1. Introduction

By now, cancer is considered and characterized as a new not completely understood disorder. Despite fast and deep developments in research through last decades, cancer issue is still remaining a worldwide problem. Epidemiological findings show after hearth disease; cancer kinds were considered as the second common factor of death (Jemal *et al*. 2007). As a pathogen S.aureus has the capability to lead a wide range of human threatening life including skin infections, toxic shock syndrome and necrotizing pneumonia (Monecke *et al*. 2011). Streptomycin is an antibiotic against bacteria attributed to amino-glycosides drug group derived from Streptomyces griseus (Watson 2015). Some reports were also demonstrates good anti cancer activity for such pharmaceutical dosage form. It's toxic effect was demonstrated that may lead to nephrotoxicity and neuroparalysis depending such parameters like dosage (Helms and Quan 2006). Dendrimers were characterized as highly branched macromolecules, sometimes called nanopolymeric biomaterials. Their structure consists of three moieties: a central core, repetitive branching units and terminal groups which change their structures to modifiable surface groups. The generation of dendrimers (surface layers) were conventionally defined by the number of repetitive branching units (Lee *et al*. 2005, Svenson and Tomalia 2012). Due to controllable size and shape, dendrimers draw attention to drug delivery and nanomedicine application (Kalomiraki *et al*. 2016). Drugs could be loaded on dendrimer's cavities or bound to external group of dendrimer's surface through hydrophobic or electrostatic even or drug-surface covalently interactions (Kesharwani *et al*. 2014). The broad spectrum of anticancer and antimicrobial drugs faces with two major difficulties. First their inefficiency in aquatic medium and the other one is their toxicity effects regarding lack of tumor specify. Dendrimers were able to overcome these difficulties significantly when they use in drug delivery system (van Dongen *et al*. 2014, Zhong *et al*. 2016). In current experiments dendrimer based-streptomycine delivery system (Anionic Linear Globular Dendrimer second generation G2- Streptomycin) was applied to improve anticancer and antimicrobial effect of Streptomycin against Breast cancer and S.aureus respectively, toxicity parameters as well as mechanism of cancer cell death were also investigated.

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2. Materials and methods

2.1 Determination of Streptomycin –G2 dendrimer loading

To assess the structure and distribution size of conjugation between G2 dendrimer and Streptomycin dynamic light scattering (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK) were applied. As well as G2 dendrimer and G2 dendrimer - Streptomycin were prepared with concentration of 100 micomolar in distilled twice water. One drop was put on lamella and placed in desiccator for draying. Then two-dimensional image was taken by the AFM microscope.

2.2 Chemical synthesis of the streptomycin –G2 dendrimer loading

Anionic linear-globular dendrimer G2 was synthesized according to the method previously reported (Assadi *et al*. 2016). In detail, 1 mL (3.7 mmol) polyethylene glycol (PEG) 600 (Merck, Darmstadt, Germany) solved in 10 mL Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany). Then, 0.75 g (2*3.7 mmol) N, N'-Dicyclohexylcarbodiimide (DCC) (Merck, Darmstadt, Germany) was added to the solution. The reaction was continued for 30 mins at room temperature while stirring. 0.71 g (2*3.7 mmol) citric acid (Merck, Darmstadt, Germany) was then added. The reaction remained stirred at room temperature for 1 h. Afterwards, 2.25 g (6*3.7 mmol) DCC and 5 mL DMSO were added and the reaction was continued under the above-mentioned conditions for about 15 mins. Finally, 2.1 g (6*3.7 mmol) citric acid was added and the reaction was continued for 1 week at room temperature, while stirring. Then, The G2 dendrimer was filtered. Purification was done by applying a Sephadex G-50 fine column (GE Healthcare Life Sciences, UK). Subsequently 500 mg Streptomycin (Merck, Darmstadt, Germany) in water was added dropwise to the solution containing functionalized dendrimer at the presence of 10 mmole EDC again and the reaction mixture was stirred at room temperature for at least one day.

2.3 In vitro cell culture

MCF-7 cell line was delivered from the Pasteur Institute of Iran in a flask. After incubation, in an incubator containing CO2 for 2 days, cells were passaged in two 75 ml flasks. For this purpose, the previous cell culture media was discarded. Then, the cells were rinsed with PBS. With adding 5 ml Trypsin, the cells were separated with 5-minute incubation in 370C. The residual cells were completely separated with pipetting as well, after adding 10 ml cell culture media containing FBS, cells were poured into a 15-ml falcon to neutralize Trypsin, 4 ml RPMI cell media containing FBS %10 and p/s %1 were added to the cells and it was completely mixed. Then, 2 ml of the cell suspension and 8 ml of the cell media was poured into two 75 ml flasks, and was left in the CO2 incubator.

2.4 In vitro apoptosis necrosis assay

An Annexin V-Propidium iodide staining kit was consumed to assess apoptosis according to the manufacturer's instruction. MCF-7 cell line (5000 cells/well) was used for the cell viability test. The cells were incubated with different amount of conjugation and same amount of Streptomycin for 48 hours with untreated cells as a positive control. Each concentration was tested in duplicate.

2.5 Real-time PCR with SYBR green A SYBR Green Real time

To investigate the expression of Bax and Bcl-2 gene in MCF-7 cell line quantitative PCR was done. RN easy Plus Mini Kit (Qiagen) was used in order to extract Total cellular RNA from the treated and untreated cells based on the manufacturer's protocol. Quanti-Tect Reverse Transcription Kit (Qiagen) was applied to isolate High quality of RNA for cDNA synthesis. based on the manufacturer's instructions. BLAST program (https://blast.ncbi.nlm.nih.gov/blast) was performed to examine Primer specificity for real-time PCR. Total volume of 20 μl reaction mixture based to the protocol of DNA master SYBR Green mix (Roche Applied Sciences) was consumed for Each real-time PCR reaction. The primer concentrations were selected 0.4 μM for genes. PCR cycling conditions consist of 10min at 95∘C, 5mins at 95∘C for cycling parameters. Melting stage: at 95∘C for 20s, 60°C for 60 s, and 95°C for 20 s in an ABI 7300 real-time PCR system (Applied Biosystems,USA) were adjusted as an Amplification stage. Comparative threshold cycle (Ct) was used to evaluate the gene expression. To provide ΔCt and ΔΔCt mean, threshold cycle (mCt) value of internal housekeeping gene (GAPDH) was taken off from mCt value of the target genes. Following Ct values were calculated as Values of each sample. The mRNA level obtained from each sample was adapted to human glyceraldehydes-3- phosphate dehydrogenase (GAPDH) mRNA level. Finally, the ratio formula (Ratio = 2 − ΔΔCt) was considered to evaluate target/control gene expression ratio.

2.6 In vitro release assessment

Assessing release study was done with dialysis bag. G2 dendrimer – Streptomycin Complex was dialyzed against an excess amount of water using membrane (pore size of dialysis bag was 500–1,000 Da) for 24 hours. The tube was immersed into a small glass beaker containing 40 mL of PBS (pH 7.4). After that,1 ml sample from beaker at prescheduled time intervals up to 96 streptomycin concentration was measured by UV-spectrophotometry at a wavelength of 256 nm.

2.7 Stability study

Measuring the stability of G2 dendrimer – Streptomycin Complex performed by dividing complex in two section. One section was kept at room temperature and another one was refrigerated at 2-8℃.The samples were taken after 1, 2,

4, 8, 12, 24, 48 and 72 h and prepared for particle size evaluation by zeta sizer.

2.8 Antibacterial activity

Evaluating. G2 dendrimer – Streptomycin Complex antimicrobial ability was performed against S.aureus using agar diffusion test. Suspensions of bacteria with a cell density equal to 0.5 McFarland ($1.5*10^8$ CFU/mL), were conveyed individually onto the surface of Muller-Hinton agar plates. After that, 0.1 ml G2 dendrimer – Streptomycin loading, 0.1 ml dendrimer G2 alone and 0.1 ml free Streptomycin were poured into the hallow wells separately which cut from agar with a sterile cork-borer in advance. With a caliper the inhibition zones around the wells were calculated 48 h post incubation at 35-37 C. All experiments were done duplicate.

2.9 MIC and MBC determination

Assessment of G2 dendrimer – Streptomycin nano-complex antimicrobial ability was performed using MIC and MBC in culture broth, based on the macro-dilution guidelines of the Clinical and Laboratory Standards Institute. After preparation a serial dilution in 5 ml of Muller-Hinton broth a variety range of G2 dendrimer – Streptomycin Complex including 0.05, 0.1,0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 μg/ml (the equivalent Streptomycin concentration was evaluated according to loading ratio). After that, 50 μl of S.aureus was adjoined to each tube to obtain a final concentration of (2*108 CFU/mL) (Ahangari *et al*. 2013). After 24 hours' incubation at 370 C., The MIC and MBC were assessed. Coevally, all abovementioned steps were repeated to evaluate MIC and MBC for a stock solution of free Streptomycin.

2.10 Statistical analysis

Statistical data analysis was performed using Prism5 and excels software (Microsoft Office 2013). For quantitative data analysis, One Way ANOVA in case of cluster comparison were applied. $P < 0.05$ was considered statistically significant by post hoc Tukey test.

3. Results

3.1 Results of determination of Ciprofloxacin –G2 dendrimer conjugate

The average size of dendrimer and Streptomycin – dendrimer were 91 and 102 nm respectively. Also Zeta potential of intact dendrimer and Streptomycin –dendrimer evaluated -3.38 and 0.544 mV respectively (Fig 2). Becoming larger in size and getting slightly positive charge in conjugation compared to the intact dendrimer confirmed loading was performed. Figs. 3(a) and (b) shows AFM microscope related to dendrimer and dendrimer-Streptomycin complex in which the particles were dispersed separately and uniformly over the lamellas.

3.2 In vitro apoptosis necrosis assay

The results of MTT assay for both Streptomycin and Streptomycin –G2 dendrimer in concentrations of 10, 50, 75 and 100 μM are shown in Fig. 4.

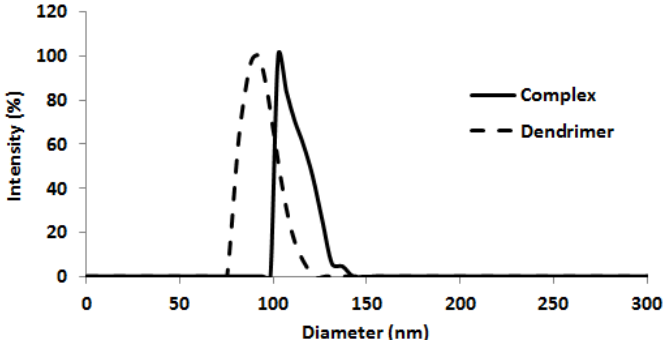


Fig. 1 Size distribution and zeta potential of G2 and Streptomycin –G2 dendrimer

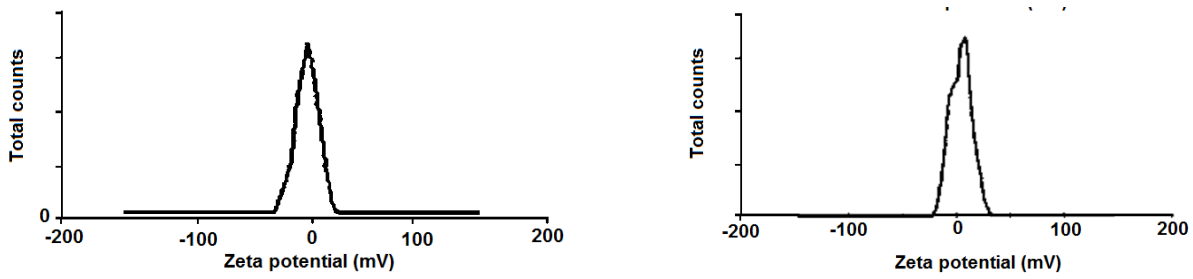
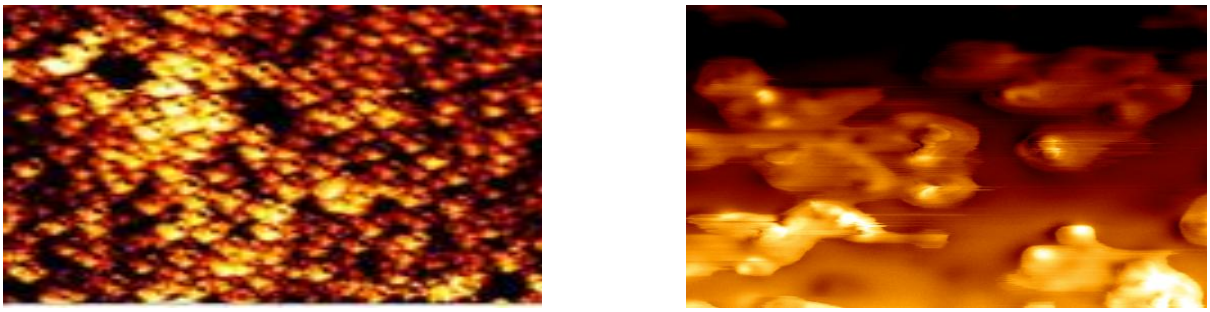


Fig. 2 Zeta potential of (A) G2 dendrimer and (B) Zeta potential distribution of Streptomycin –G2 dendrimer



(a) (b)

Fig. 3 3D AFM image of intact G2 dendrimer (a) in comparison with Streptomycin –G2 dendrimer (b)

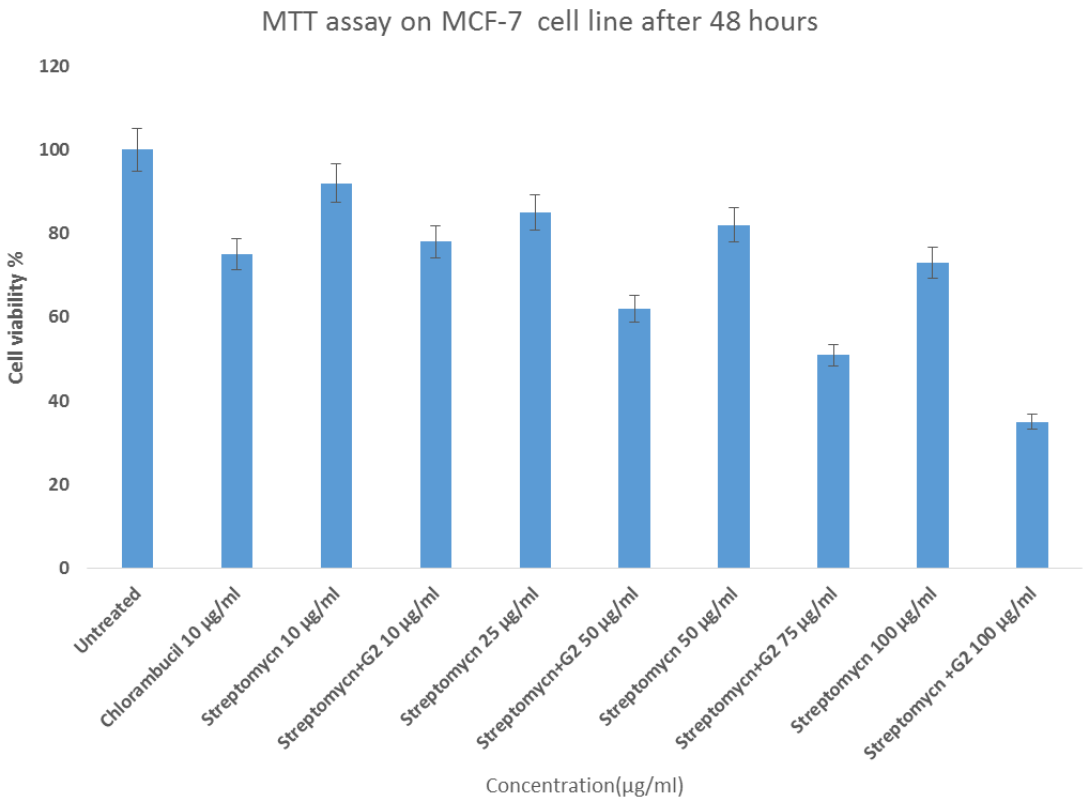


Fig. 4 Results of MTT assay on MCF-7 cells, as percentage cell viability at different concentrations, compared using Streptomycin and Streptomycin –G2 dendrimer

It is crystal clear from the figure that hiking the concentration of both the drug and conjugation caused to decreasing the cell viability. Significant statistical differences can be seen in the control with 10, 50, 75 and 100 μM of Streptomycin and Streptomycin –G2 dendrimer ($p < 0.001$). A significant difference ($p < 0.05$) was observed between the 75 and 100 μM Streptomycin and the same dose in complex. Nano drug comlex compared to drug alone in 75 and 100 μM illustrated significantly high toxicity on the cancer cell. The same conforming evidence was observed using a flow cytometry assay (Fig. 5). In order to measure the IC50 (half maximal inhibitory concentration) of these samples, linear model chart was performed. The IC50 of Ciprofloxacin was 257 μM and IC50 of Streptomycin –G2 dendrimer was 55 μM comparing the results, it can be found that the new complex kills 50% of cancerous cells in the lower concentration compared to free Streptomycin

3.3 Analysis of apoptosis-related gene expression

It can be concluded from analysis of apoptosis-related gene expression data that mRNA level of Bax experienced marked upregulated whilst the expression of antiapoptotic Bcl-2 had relatively downregulated in cells treated with nano- drug complex in comparison with untreated control. It seems these changes may refer to apoptotic potential of new nano drug complex when MCF-7 cell line exposed to it. Consequently, the Bax/Bcl-2 ratio increased (Fig. 6).

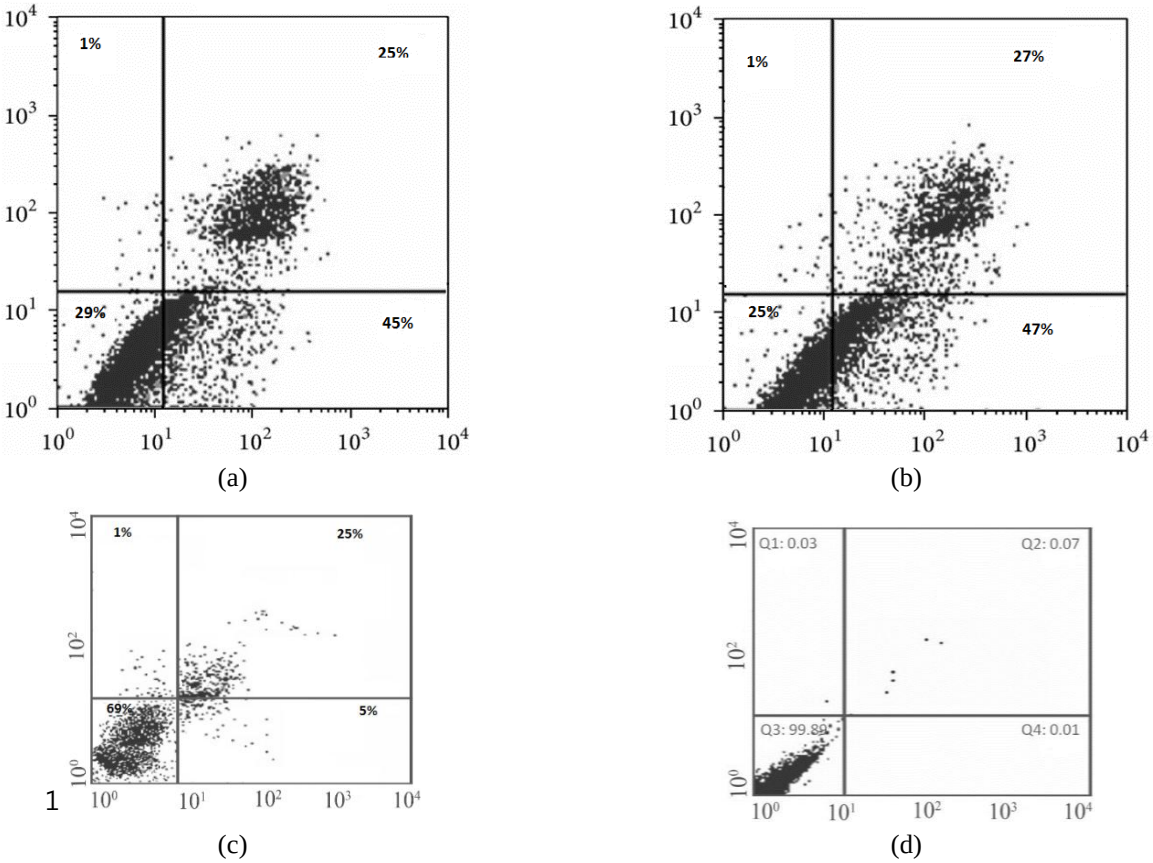


Fig. 5 Dot plots of Annexin V-propidium iodide staining are shown in (A) MCF-7 cells treated with 100 μg/mLG2 dendrimer- Streptomycin after 24 hours; (B) MCF-7 cells treated with 100 μg/mLG2 dendrimer- Streptomycin after 48 hours (C) MCF-7 cells treated with 100 μg/mL Streptomycin; and (D) untreated MCF-7 cells

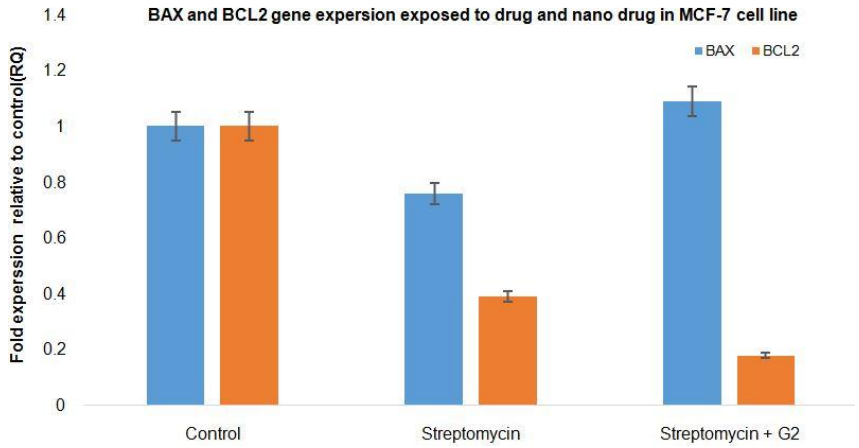


Fig. 6 The effect of Streptomycin and G2 dendrimer- Streptomycin on Bax and Bcl-2 gene expression

3.4 In vitro release of streptomycin from the drug loaded on dendrimer

Streptomycin release from complex was measured through UV-spectrophotometry (Fig. 7). It could be seen a fast drug release after 20 h in which 50 percent of drug was released from complex. A regular hiking pattern would be seen after 20 h reached to 100 percent after 80 h. This releasing model is desirable one for a controlled drug release system.

3.5 Stability of Streptomycin –dendrimer G2

The results came from stability test of complex showed the complex unaltered during 72 h without any sensitivity toward temperature (Fig. 8).

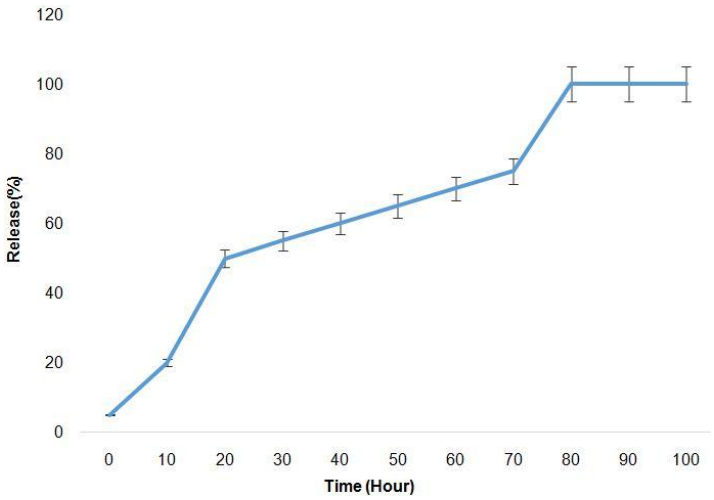


Fig. 7 In vitro release profile of Streptomycin from Streptomycin-dendrimer G2 complex

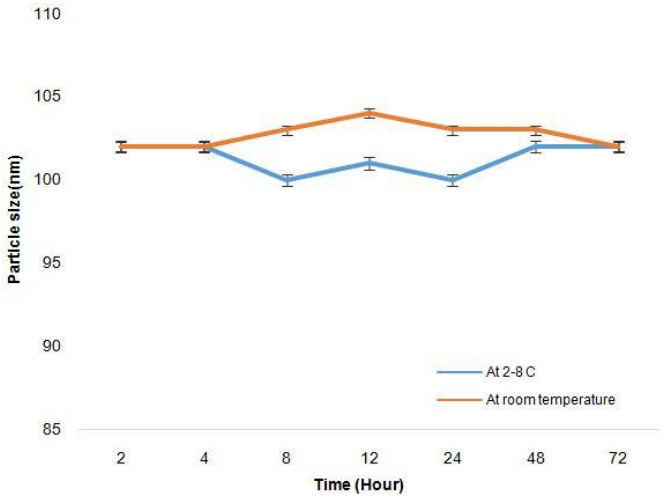


Fig. 8 The stability test of Streptomycin-dendrimer G2 complex

3.6 Antimicrobial activity

According to obtained data as illustrated in Table 1, Streptomycin –G2 dendrimer antimicrobial ability outweighed free Streptomycin ($P < 0.05$) following agar diffusion method.

3.7 MIC and MBC determination

Based on MIC and MBC determination results it could be seen that a significant difference between Streptomycin –G2 dendrimer and Streptomycin alone against S. aureus displayed in Table 2. This improvement would come from Bactericidal potential attributed to dendrimer G2.

Table 1 Antimicrobial susceptibility study by measurement of bacterial inhibition zone around the wells

Groups	Inhabitation zone around the well (mm)
Streptomycin	20/86 ± 1.04
Streptomycin-dendrimer G2	24/02 ± 1.12
dendrimer G2	15/75 ± 0.95

Table 2 MIC and MBC for free streptomycin versus streptomycin-dendrimer G2 complex

	Free Streptomycin (μg/ml)	Streptomycin-dendrimer G2 (μg/ml)
MIC	0.2	0.05
MBC	0.4	0.1

4. Discussion

Some new drugs in markets have been faced with a series of inefficiency like low aqueous solubility and short half-life. Nanoparticles have used to overcome these difficulties because of their ability to modify the basic properties of drug solubility, half-life, biocompatibility and

its release characteristics (Hu *et al*. 2010). Dendrimers are polymeric architectures that are diagnosed by its differentiate and versatile structure used in drug delivery. These biodegradable nanostructured macromolecules have shown their potential abilities in entrapping and/or conjugating high molecular weight hydrophilic/hydrophobic entities by host-guest interactions and covalent bonding (prodrug approach) respectively (Alavidjeh *et al*. 2010, D'emanuele and Attwood 2005). Recently polymers have been used largely in drug delivery since pharmacokinetics, biodistribution and controlled release of the drug improved. improved pharmacokinetics, biodistribution and controlled release of the drug (Allen and Cullis 2004). Thanks to their above mentioned properties, dendrimers have drawn attention in biological application especially in drug delivery compared to traditional polymers (Duncan and Izzo 2005, Soto-Castro *et al*. 2012, Tomalia 2005). Therefore, this study was focused on loading of Anionic linear globular dendrimer G2 on Streptomycin by considering dendrimer properties such as biocompatibility and biodegradability to improve anticancer and antimicrobial efficacy of drug. Anticancer dendrimer-based drugs have shown efficient results against cancer cells both in vitro and vivo because of their especial features. In a research was performed on anticancer efficiency of Anticancer dendrimer-based drugs, it is concluded that Anionic linear globular dendrimer G2 conjugated with cisplatin [cis-diaminedichloroplatinum; (CDDP)] has extremely anticancer potency rather than drug alone when it exposed to several cancer cell lines (Haririan *et al*. 2010). The successful synthesis of the Streptomycin –G2 dendrimer confirmed by AFM and DLS.MTT (in vitro cytotoxicity) assay disclosed that Streptomycin –G2 dendrimer was more toxic against cancer than drug alone following interaction of Streptomycin –G2 dendrimer 48 h with MCF-7 cell line. Apoptotic and/or necrotic cells were examined using the Annexin V-propidium iodide staining kit according to the manufacturer's protocols. The percentage of apoptotic cells was evaluated by flow cytometry. Apoptosis is a mode of cell death that occurs under normal physiological conditions and in which the cell is an active participant in its own death (Salehi *et al*. 2016). Compared with Streptomycin, the effect of Streptomycin –G2 dendrimer on the apoptotic cells was stronger. In the current study, we delved in more accurate analysis over apoptosis mechanism by doing real-time PCR and it was concluded that Bax/Bcl2 genes ratio was extremely high as an apoptosis criterion. The elongation release drug from complex for 72 h showed a controlled-release of the loaded drug. Complex size remained unaltered after 72 h which is rather efficient from stability test aspect. Antimicrobial tests evidence demonstrated that complex was more bactericidal than free drug. It could be originated the fact that Streptomycin molecules became denser when they loaded on dendrimer surface. Subsequently, membrane permeability was increased absorption causing Streptomycin molecules to enter the bacteria. Consequently, potassium ions leakage leads to decomposition of the bacterial membrane (Chen and Cooper 2002).

5. Conclusions

It is obvious that more studies need to be done to understand the whole characteristics of the complex. However, these advantages include biocompatibility and biodegradability, anticancer potency compared to Streptomycin is very interesting for the Streptomycin –G2 dendrimer complex as a new drug delivery system.

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