Antibacterial effect of TiO₂ modified with poly-amidoamine dendrimer – G3 on S. aureus and E. coli in aqueous solutions

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ABSTRACT

This study investigated bacterial removal using TiO₂ nanoparticles (NPs) modified with poly-amidoamine dendrimer macromolecule (PAMAM, G3). The PAMAM G3/TiO₂ (nanohybrid) was used to specify antibacterial properties via broth microdilution (MBC-Minimum Bactericidal Concentration and MIC-Minimum Inhibitory Concentration-determination), paper disc diffusion, and surface plate count methods. The nanohybrid was characterized via the different techniques. The effects of different factors including initial bacteria count, run time, solution pH, and the nanohybrid concentration were studied. The nanohybrid cytotoxicity was studied on AGS and MKN45 cells line by MTT assay. It was revealed that the nanohybrid was effective in intercepting both bacterial strains growth. The MIC value for S. aureus and E. coli were determined to be 4 and 2 μg/mL, respectively. The MBC value for both strains were calculated to be 32 μg/mL. The results showed removal efficiency of 100% for S. aureus and E. coli bacteria in optimum situation. The decrease in cell viability in the dosage of 32 μg/mL after 72 h treatment for AGS and MKN45 cells line were shown to be 6.2 and 4.6%, respectively. The nanohybrid was able to decrease the S. aureus and E. coli count in solution, which meets the drinking water criterions aligned with WHO guidelines.

Key words: antibacterial, biocompatible, cytotoxicity, dendrimer, macromolecule, microdilution, nanohybrid

HIGHLIGHTS

• A novel poly (amidoamine) generation 3-functionalized TiO₂ nanoparticle was prepared.
• The antibacterial activity was displayed with particles of a nano size which exhibited a distinct homogenous morphology.
• Super high removal efficiency was achieved for bacteria in aqueous solution: E. coli and S. aureus.
• FE-SEM analysis revealed morphological variations and the mechanism of killing and trapping the bacteria by nanocomposite.
• In the MIC and MBC values range, the cytotoxicity effect of nanocomposite on AGS cell line was relatively lower.
1. INTRODUCTION

According to the World Health Organization (WHO), it is estimated that annually one million people lose their life due to diarrhea, mostly children under five years old, as a result of unsafe drinking water (Kouhsari et al. 2018). Diarrhea is the consequence of microorganisms in unsafe water, highlighting the importance of water quality control which will have noticeable achievements in disease control (Santika et al. 2020). Microbial safety in the treatment of wastewater needs to be considered as well. In order to deactivate microorganisms, some chemical disinfectants such as ozone, chlorine and chloramines are used (Tang et al. 2019). Sometimes, these disinfectants combined with other elements in water and harmful combinations or carcinogenic disinfection byproducts get created (Srivastav et al. 2020). So, high dosage of disinfectants will be required since some pathogens are resistant to them. Some state-of-art disinfectants have been suggested to be replaced with the old ones like chitosan (Li et al. 2019), silver nanoparticles (Deshmukh et al. 2019), copper nanoparticles (Lv et al. 2018), carbon nanotubes (Morsi et al. 2017) and AgCl-TiO₂ (Uz et al. 2020). During recent years, cationic dendrimers have shown great performance. They are branched polymeric nanoparticles and have been widely used in biomedical applications such as gene delivery and drugs. This is because their physicochemical properties are easily controlled (Nazari et al. 2019). The cellular membrane bypass can be controlled by their size, shape and surface charge which form complexes with deoxyribonucleic acid (DNA) as a result (Mukherjee et al. 2010). Because of this accurate control over the physicochemical properties, dendrimers are unique among other nanoparticles, such as polymer and surfactant micelles, and are highly considered in biomedical applications (Fox et al. 2018). Dendrimers are made up of layers of dendrons radiating from a central initiator core.

Each layer is termed as a generation (Golshan et al. 2020). Diver types of dendrimers, over 100 types, are available with different initiator cores such as carbon, nitrogen and phosphorus and also various branching units and multiplicities (Fox et al. 2018). Poly-amidoamine (PAMAM) dendrimers are assumed to be a complete family to study that initially was investigated by Tomalia et al. in the 1980s (Scott et al. 2005). They are easily accessible and low cost and their toxicity was widely studied in fields of drug delivery, gene transfection, and imaging (Maleki et al. 2016). Antimicrobial properties of dendrimer derivatives have been considered substantially in recent years. This property is attributed to the electrostatic interaction between the cationic dendrimer and the anionic bacteria cell surface (Rastegar et al. 2017). During this interaction, cell death occurs due to the disruption of lipid bilayer. Considering localization in specific organs, low toxicity and high speed, dendrimer biocides become more useful (Gholami et al. 2016).

This research aimed at producing and using a PAMAM/TiO₂ to remove Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus) bacteria from aqueous solution. There were some reasons to choose TiO₂ NPs including wide surface area, great mechanical strength and its photocatalytic properties. Some parameters influencing the quality of removal were also investigated, i.e. number of initial bacteria, pH, required time and dosage of the nanohybrid.
2. MATERIALS AND METHODS

2.1. Material
PAMAM dendrimer (G3, dissolved in 10% of methanol, CAS No. 202009-64-1) was obtained from Sigma Aldrich. TiO₂ with 99% purity was purchased from Merck (Germany, CAS No. 13463-67-7). The diameter of PAMAM-G3 is 3.6 nm, molecular weight equal to 6848.79 g/mol and the number of terminal amine groups is 32. The structure of PAMAM-G3 is also displayed in Figure 1.

2.2. Production of PAMAM/TiO₂ nanohybrid
In order to produce the PAMAM/TiO₂, 1 g of dendrimer was dissolved in 20 mL methanol, which has diluted with 1 L of distilled water. Then TiO₂ was entered into the solution slowly. When all of the TiO₂ nanoparticles were added, the product solution had a mass ratio of 1:99. In order to remove all moisture from PAMAM/TiO₂, it was dried at 105 °C for 2 h (Vacuum Oven, XU490), after ultrasonic cleaning for about 2 h (ultrasonic cleaner, VWR, 50 kHz, 150 W).

2.3. Properties of PAMAM/TiO₂
By means of Perkin-Elmer Frontier spectrophotometer in the wavenumber range of 450–4000 cm⁻¹, the FTIR spectra of the PAMAM/TiO₂ was analyzed. Using a PW 1800 X-ray (Philips) diffractometer, X-ray diffraction (XRD) analysis was also carried out. Transmission electron microscopy (TEM-Philips CM 30) was utilized with an accelerating voltage (200 kV). By spreading a small drop containing PAMAM/TiO₂ on holey carbon TEM grids, the samples were gained and dried for 2 h in 25 °C (Nazari et al. 2019). The morphology and size of the samples were obtained and analyzed using electron microscope scanning (SEM, Zeiss-Germany). A surface area analyzer (Quanta Chrome Instruments, Nova 2000, USA and FL) performed BET surface in which nitrogen was used as the adsorption gas. Under a nitrogen atmosphere, the samples were gradually heated for 4 h at 500 °C (Maleki et al. 2016).

2.4. Bacterial properties and culture medium
Two strains were selected in this research which were S. aureus (ATCC 25923) and E. coli (ATCC 25922) as indicators of gram-negative and gram-positive bacteria, respectively. They were provided from the Pasteur Institute (Iran, Tehran). Culture medias (nutrient broth, Mannitol salt agar and MacConkey agar) were purchased from Merck (Germany). Then, nutrient...
broth was used to develop the pure cultures of each bacterium for 24 h at 37 °C (condition was aerobic). A sterile inoculating loop was used to put the suspension of \( S.\ aureus \) and \( E.\ coli \) onto Mannitol salt agar and MacConkey agar, respectively and incubated at 37 °C for 48 h (Federation & Association 2005).

### 2.5. Evaluation of antibacterial activity

Various techniques encompassing broth micro dilution (MIC and MBC determination), paper-disc diffusion based on the clinical laboratory standards institute (CLSI) procedure (Wayne 2011), and surface plate count (Colony Forming Unit-CFU/mL) in phosphate buffer solution (PBS) (0.01 mmol/L) were used to evaluate the activities (Federation & Association 2005).

#### 2.5.1. MBC and MIC testing

The lowest concentration of a particular bacterium which prevents its growth is called MIC and is done by a micro dilution method proposed by CLSI (Wayne 2011) in 96 well cultured plates. The detailed procedure was described in previous studies (Rastegar et al. 2017).

#### 2.5.2. Paper-disc diffusion method

We have previously discussed this method (Gholami et al. 2016; Rastegar et al. 2017). A dried disc containing 40 μl PAMAM G3/TiO\(_2\) with a concentration of 32 μg/mL was located on the plate. Then they were incubated for 24 h at 37 °C and a caliper was used to measure the inhibition zone (IZ). To compare, a blank disc was implemented without the PAMAM G3/TiO\(_2\) (distilled water as control sample).

#### 2.5.3. Surface plate count method

We used 50 mL sterilized PBS (0.01 mmol/L) to disperse PAMAM G3/TiO\(_2\) NPs solution by adding 3.33, 33.33 and 333.3 μL of bacterial culture in peptone broth (1.5 × 10\(^8\) CFU/mL), initial bacteria count was 10\(^5\), 10\(^4\) and 10\(^3\) CFU/mL, respectively. Some characteristics like the effects of initial pH, PAMAM G3/TiO\(_2\) concentrations, initial bacteria count and required time were investigated and are listed in Table 1 in various conditions. We managed to gather 100 μL aliquots of the sample and diluted to 10-fold concentration gradient serially with a PBS (0.01 mM) solution. Then, 100 μL of supernatant from \( S.\ aureus \) and \( E.\ coli \) samples were streaked on mannitol salt agar (MSA) and MacConkey agar medium, respectively. In order to identify the survival percentage during treatment, the colonies were counted (CFU/mL) by using Equation (1). As the control, we assessed the primary bacterial solution:

\[
E = \left( \frac{C_{in} - C_{out}}{C_{in}} \right) \times 100
\]  

where \( C_{in} \) is primary count, \( C_{out} \) is final count.

### 2.6. Cytotoxicity assay

The cytotoxicity of PAMAM G3/TiO\(_2\) on AGS and MKN-45 cell lines (human caucasian gastric adenocarcinoma) were evaluated using MTT assay (Niapour et al. 2015). The AGS and MKN cell lines were obtained from the national cell bank of Iran.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Range of operating parameters for ( E.\ coli ) and ( S.\ aureus ) abatement by PAMAM/TiO(_2) NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of experiments</td>
<td>initial pH</td>
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<tr>
<td>Effect of initial pH</td>
<td>5–8</td>
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<tr>
<td>Effect of PAMAM/TiO(_2) concentration (μg/ml)</td>
<td>7</td>
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<td>Effect of run time (min)</td>
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<td>Effect of ( E.\ coli ) count (CFU/mL)</td>
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<td>Effect of ( S.\ aureus ) count (CFU/mL)</td>
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(Pasteur Institute of Iran). Cells at the density of $5 \times 10^3$ were plated into wells ($n = 96$). The following day, cells were treated with increasing concentrations (1, 4, 8, 16, 32, 64, 128, 256, 512 and 1,024 $\mu$g/mL) of PAMAM G3/TiO$_2$ or 30 $\mu$g/mL cisplatin as a positive control for 48 h. The medium of each well were then replaced with 0.25 mg/mL MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma: M2128) and incubated for 4 h at 37 °C. Dimethyl sulfoxide (DMSO; Sigma: D4540) was used to solve formazan crystals. Finally, the absorbance was measured at 570 nm using ELISA reader (Synergy HT, BioTek).

2.7. Data analysis
All measurements were performed three times in two replicates and the mean amounts were represented.

3. RESULTS AND DISCUSSION
3.1. Characterization of PAMAM/TiO$_2$ nanohybrid
We used FTIR spectra to analyze the PAMAM/TiO$_2$. Based on the results (Figure 2), C-H bond appeared at 2972 cm$^{-1}$, as a peak. C=O amide stretching group, C-N amide stretching, and C-N-H polyamide bending resulted in maximum points at 1648, 1546, and 1279 cm$^{-1}$, respectively. The asymmetric and H-CH bending bond caused maximum points of 1459, 1428, and 1349 cm$^{-1}$. Ti-O-C bonds caused peaks at 1092 and 1046 cm$^{-1}$. The dendrimer chemical bond and TiO$_2$ was related to Ti-O-C bonds.

An inverse relation between TiO$_2$ NPs and dendrimers was observed as if one was positive, the other will be negative which resulted in a strong electrostatic interaction (Maleki et al. 2016). The effective functionalization of TiO$_2$ with amine terminated PAMAM-G3 dendrimer was validated by the presence of the bending vibration of $-\text{NH}_2$ group at 3422 cm$^{-1}$ and $-\text{CO-NH}$-group at 1648 and 1546 cm$^{-1}$. The results of the XRD analysis spectra was presented in Figure 3. The peaks at $2\theta = 25.4$, 37.3, 48.0, 54.3 and 62.8 were observed by analyzing peaks of TiO$_2$ NPs in previous studies (Akilavasan et al. 2015; Maleki et al. 2016). All the peaks were shown by the PAMAM/TiO$_2$ including to the TiO$_2$ NPs. However, no refraction peaks of PAMAM were seen. Chemical and inter molecular forces had loaded PAMAM on TiO$_2$ NPs as the results revealed (Peng et al. 2016).

TEM analysis was used to identify the diameter and morphology of the PAMAM/TiO$_2$. It should be noted that the shapes of PAMAM/TiO$_2$ were spherical/spheroidal with smooth surface and the mean diameter of 50 nm (Figure 4(a)–(c)). The SEM image (Figure 4(d) and (e)) validates the size and shape. Energy Dispersive X-ray (EDX) was used to analyze elemental analysis of the PAMAM/TiO$_2$ NPs. TiO$_2$ and PAMAM-G3 dendrimer in the PAMAM/TiO$_2$ nanohybrid appeared by the existence

![Figure 2](http://iwaponline.com/wst/article-pdf/85/2/605/998201/wst085020605.pdf)  
**Figure 2** | FTIR patterns of PAMAM/TiO$_2$ nanohybrid.
of Ti and Si signals and N and C elements, respectively. The percentage of O, N, C, Si and Ti was illustrated to be 42.07, 0.1, 2.58, 6.8 and 50.33% (Figure 4(f)), respectively by which the connection of dendrimers to TiO$_2$ NPs surface was validated.

BET technique and nitrogen adsorption/desorption isotherms were implemented to characterize the surface of TiO$_2$ and PAMAM/TiO$_2$. The findings (Table 2) revealed that surface area and pore volume of amino functionalized by PAMAM/TiO$_2$ were lower than TiO$_2$ because of the introduction of organic functional groups into the pores resulting in pore size reduction and material density increase. Narrowing, opening and blockage of TiO$_2$ pores is due to attached PAMAM molecules.

**Figure 3** | XRD pattern of PAMAM/TiO$_2$ nanohybrid.

**Figure 4** | TEM (a, b and c), FE-SEM (d, e), and corresponding EDX analyses of PAMAM/TiO$_2$. 
3.2. Bactericidal performance of PAMAM/TiO\textsubscript{2} NPs

3.2.1. MIC and MBC of PAMAM/TiO\textsubscript{2} NPs

MIC values were calculated to be 2 $\mu$g/mL for \textit{E. coli} and 4 $\mu$g/mL for \textit{S. aureus}. Moreover, an MBC value of 32 $\mu$g/mL was revealed for both. According to these results, it could be claimed that PAMAM/TiO\textsubscript{2} has antibacterial activities or effects.

It acts using the following steps: (1) absorption of ammonium groups, (2) destroying the cell wall, (3) cytoplasmic membranes grafting, (4) deforming the membranes, (5) releasing cytoplasmic items like DNA, RNA and K\textsuperscript{+}, and (6) stopping bacterial activation (Chen et al. 2000; Nazari et al. 2019).

The antibacterial impacts of PAMAM-G7 and G6 dendrimers on multidrug-resistant strains was studied by Gholami et al. (2017) and Rastegar et al. (2017). Some polymers with antibacterial activities were PAMAM dendron-modified magnetic NPs, amino-functionalized magnetite/silica, and quaternized N, N dimethylaminoethyl methacrylate (Arakaki et al. 2016).

3.2.2. Bacteria inhibition zone

As we observed, the obtained MBC concentration for both bacteria was 32 $\mu$g/mL so that the same value was used when working with the paper-disc diffusion method. In Figure 5(a) and (b), photographs of the results are presented. Ultra-pure water was used as control sample. \textit{E. coli} was found to be more sensitive against PAMAM/TiO\textsubscript{2} than \textit{S. aureus} (IZ was recorded as 40 and 34 mm respectively), this is because gram-negative bacteria have got thinner peptidoglycan walls compared to gram-positive (Wang et al. 2020). Gram-negative bacteria absorb more amine in their surfaces than gram-positive bacteria so the charge density of gram negative is higher (Charles et al. 2012). The obtained results were supported by previous studies (Nazari et al. 2019; Roig-Molina et al. 2019).

3.2.3. Experimental parameters affecting bacterial removal

3.2.3.1. First stage pH. Amino modified NPs performance was seriously affected by pH and a series of tests were conducted based on the parameters declared in Table 1. The pH and bacterial removal showed a positive relationship. As we increased the primary pH from 5 to 7, the removal effectiveness of \textit{S. aureus} and \textit{E. coli} dramatically increased from 65 and 70\% to 100\%, respectively (Figure 6). A negligible impact was shown when increasing pH from 7 to 8. Other factors such as bacteria surface features, surface hydrophobicity and surface charge influence the removal process as well (Goswami & Pugazhenthi 2020). PAMAM/TiO\textsubscript{2} surface with positive charge in interaction with bacteria with negative charge leads to removal due to electrostatic power. (Rastegar et al. 2017). The highest abatement efficiency was seen at pH 7, so it was considered as a favorable point in this study.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Materials & Specific surface area (m\textsuperscript{2}/g) & Pore volume (cm\textsuperscript{3}/g) \\
\hline
\textit{TiO}_2 & 120.8 & 32.7 \\
\textit{PAMAM/TiO}_2 & 75.5 & 0.34 \\
\hline
\end{tabular}
\caption{The specific surface area and pore volume data of TiO\textsubscript{2} and PAMAM}
\end{table}

\textbf{Figure 5} | Antibacterial activity of PAMAM/TiO\textsubscript{2} against \textit{E. coli} (a) and \textit{S. aureus} (b) bacteria.
3.2.3.2. The effect of PAMAM/TiO₂ concentration. Based on the parameters in Table 1, a number of experiments were performed in order to measure the effects of PAMAM/TiO₂ concentration. The 1 h removal efficiency of both bacteria is shown in Figure 7. As the concentration was increased from 2 to 32 μg/mL, the survival rate of *S. aureus* and *E. coli* decreased from 400 and 340 CFU/mL to zero. So, it could be concluded that PAMAM/TiO₂ have great antibacterial activities against *S. aureus* and *E. coli*. This finding was also consistent with previous studies (Zhan et al. 2014).

3.2.3.3. Effect of run time. In the first 5 min, survival percentage of *S. aureus* and *E. coli* were obtained to be 51.2 and 45.6%, respectively. In 1 h, no alive bacteria were seen according to WHO guidelines for drinking water (Kumar & Puri 2012). More PAMAM/TiO₂ interaction with bacteria were observed as the time of process was increased (Figure 8). Since, the survival percentage of *S. aureus* and *E. coli* in 50 min was almost 2%, it is not appropriate for drinking water according to WHO guidelines (Edition 2011).

3.2.3.4. Effect of primary bacteria count. Figure 9 displays the remaining bacterial cells versus the primary bacteria counts. As the primary bacteria count has been increased, the remaining bacterial cells increased as well. Survival percentage was increased from zero to 70 and 56 CFU/mL for *S. aureus* and *E. coli*, respectively, while the count was raised from 10³ to 10⁴ CFU/mL. The remaining bacterial cells dramatically increased to 270 and 240 CFU/mL for *S. aureus* and *E. coli*, respectively after the increment (10⁴–10⁵ CFU/mL). According to the results obtained, PAMAM/TiO₂ failed to absorb all
bacterial cells since the number of it was greater than bacterial cells in bacterial low level. So it is clear that the removal process improved in lower amounts of bacteria.

3.3. Morphological variations in E. coli and S. aureus cells

FE-SEM was conducted to find out more about possible mechanisms for stopping the growth of E. coli and S. aureus caused by PAMAM/TiO₂. The images obtained from the test are shown in Figure 10, both in the absence and presence of PAMAM/TiO₂ in favorable conditions and revealed that bacteria were effectively caught by PAMAM/TiO₂. One of the effective ways was the binding of the PAMAM/TiO₂ to the cells surface. At pH 5–9, the surfaces of these bacteria are negatively charged. Hence, electrostatic force on the PAMAM/TiO₂ surface quickly absorbs these bacteria. Figure 10(a)–(c) shows that plenty of S. aureus (right side, spherical shapes) and E. coli (left side, a rod-like shapes) cells are in the bacterial solution before the antibacterial action. After 1 h, nearly all bacteria were died. The structure of control sample (S. aureus and E. coli cells incubated in PAMAM/TiO₂ -free) is demonstrated in Figure 10(a)–(c).

The cells in Figure 10(d)–(f) were totally damaged by PAMAM/TiO₂. Furthermore, PAMAM/TiO₂ penetrated into the membrane and destroyed its structure and had better for them to have smaller sizes. Due to the positive charge of amines in their surfaces, they are widely used in cell removal (Zhan et al. 2014).

3.4. Cytotoxicity studies in laboratory conditions

In order to investigate the effect of PAMAM/TiO₂ on AGS and MKN-45 cells line, a cytotoxicity test was conducted. The MTT findings are shown in Figure 11. The increment of concentration enhanced the cytotoxic impact on target cells. A
PAMAM/TiO₂ concentration increase from 1 to 1024 μg/mL resulted in cell viability decline for AGS from 90.72 to 77.1% and MKN from 94.4 to 83.5%. The cytotoxicity of 6th and 7th generations of PAMAM dendrimers were investigated with various doses and incubation time. Generally, an increase in dosage and time of exposure resulted in higher cell viability loss (Rastegar et al. 2017; Nazari et al. 2019). The MIC for both S. aureus (8 μg/mL) and E. coli (4 μg/mL) bacteria showed that PAMAM/TiO₂ had a toxic effect on selected bacteria at reasonably lower concentrations. In these concentrations, nanoscale holes are usually formed by PAMAM dendrimers in prokaryotic membranes. Meanwhile, cytotoxicity results showed that more than 80% of examined cells were alive at 32 μg/mL PAMAM/TiO₂. This indicates the sensitivity of bacteria to PAMAM/TiO₂. These findings could be related to the density of negative charges on the surface of the bacteria.

**Figure 10** | FE-SEM images of E. coli (a and b) and S. aureus (c) from control samples, and E. coli (f and e) and S. aureus (d) obtained after 60 min run time with PAMAM/TiO₂ NPs, respectively.

**Figure 11** | Assessment of PAMAM/TiO₂ NPs cytotoxicity toward AGS and MKN cells measured by MTT metabolic activity assay in 72 h with 30 μg/mL cisplatin as the positive control. Evaluation of percent viability of AGS and MKN cells after treating with PAMAM/TiO₂ at various concentrations was.
which may provide an affinity for the higher positive charge of PAMAM/TiO\textsubscript{2} nanoparticle. This process will cause bacterial death (Hong et al. 2006).

4. CONCLUSIONS
The aim of this study was to provide a convenient method to remove \textit{S. aureus} and \textit{E. coli} bacteria using PAMAM-G3 functionalized TiO\textsubscript{2} as fast as possible. The results revealed that sufficient run time to completely (100\%) remove the bacteria is 1 h for both groups. pH 7, PAMAM/TiO\textsubscript{2} dosage of 32 \(\mu\)g/mL, and primary bacteria count of 10\textsuperscript{3} CFU/mL were illustrated as the optimum points. In the MIC and MBC amount range, the PAMAM-G3/TiO\textsubscript{2} cytotoxicity effect on AGS and MKN cell line was relatively lower. So, for maintaining water safety, PAMAM-G3/TiO\textsubscript{2} is proved to be an appropriate option.

ACKNOWLEDGEMENTS
This paper is extracted from a master thesis approved in the Department of Environmental Health Engineering. The project is financially supported by the Vice Chancellor for Research and Technology of Ardabil University of Medical Sciences (Grant No. IR.ARUMS.REC.1398.210).

CONFLICTS OF INTEREST
There is no conflict of interest.

DATA AVAILABILITY STATEMENT
All relevant data are included in the paper or its Supplementary Information.

REFERENCES


First received 8 October 2021; accepted in revised form 2 January 2022. Available online 7 January 2022