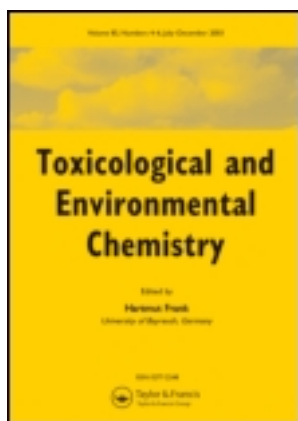


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Toxicological & Environmental Chemistry

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/gtec20>

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Zahra Bahri-Kazempour^{a b}, Khosro Abdi^c, Mojtaba Shakibaie^{a d}, Hamid Forootanfar^{a d}, Hossein Attar^d & Ahmad Reza Shahverdi^a

^a Department of Pharmaceutical Biotechnology and Biotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

^b Department of Chemical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran

^c Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

^d Pharmaceutics Research Center, Kerman University of Medical Sciences, Kerman, Iran

Accepted author version posted online: 01 Nov 2012. Published online: 19 Nov 2012.

To cite this article: Zahra Bahri-Kazempour, Khosro Abdi, Mojtaba Shakibaie, Hamid Forootanfar, Hossein Attar & Ahmad Reza Shahverdi (2013) Synthesis and antibacterial activity of a Fe₃O₄-AgCl nanocomposite against Escherichia coli, Toxicological & Environmental Chemistry, 95:1, 118-126, DOI: [10.1080/02772248.2012.745862](https://doi.org/10.1080/02772248.2012.745862)

To link to this article: <http://dx.doi.org/10.1080/02772248.2012.745862>

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Synthesis and antibacterial activity of a Fe₃O₄–AgCl nanocomposite against *Escherichia coli*

Zahra Bahri-Kazempour^{ab}, Khosro Abdi^c, Mojtaba Shakibaie^{ad},
Hamid Forootanfar^{ad}, Hossein Attar^d and Ahmad Reza Shahverdi^{a*}

^aDepartment of Pharmaceutical Biotechnology and Biotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran; ^bDepartment of Chemical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran; ^cDepartment of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran; ^dPharmaceutics Research Center, Kerman University of Medical Sciences, Kerman, Iran

(Received 29 May 2012; final version received 25 October 2012)

In this investigation, Fe₃O₄ magnetic nanoparticles (MNPs) were prepared by the alkalization of an aqueous medium containing ferrous sulfate and ferric chloride. In the next step, a Fe₃O₄–AgCl magnetic nanocomposite was fabricated by the drop-by-drop addition of silver nitrate solution into a NaCl solution containing Fe₃O₄ MNPs. All prepared nanoparticles were characterized by transition electron microscopy (TEM), X-ray diffraction (XRD), and energy-dispersive X-ray spectroscopy (EDS). Both particle types varied in size from 2.5 to 20 nm, with an average size of 7.5 nm for Fe₃O₄ MNPs and 12.5 nm for Fe₃O₄–AgCl nanocomposites. The antibacterial effect of the Fe₃O₄ MNPs and fabricated Fe₃O₄–AgCl nanocomposites against *Escherichia coli* (ATCC 35218) were investigated by conventional serial agar dilution method using the Müller–Hinton Agar medium. The minimum inhibitory concentration was 4 mg mL⁻¹ for Fe₃O₄ MNPs and 2 mg mL⁻¹ for the Fe₃O₄–AgCl magnetic nanocomposites. Time-kill course assays showed that the Fe₃O₄–AgCl magnetic nanocomposites successfully killed all inoculated bacterial cells during an exposure time of 60 min. The antibacterial activity of recycled Fe₃O₄–AgCl magnetic nanocomposites over four 60 min cycles of antibacterial treatment was further tested against *E. coli* by the colony-forming unit (CFU) method. The antibacterial efficiency of the nanocomposites was constant over two cycles of antibacterial testing.

Keywords: silver chloride; Fe₃O₄–AgCl nanocomposites; magnetic nanoparticles; *Escherichia coli*

Introduction

The antimicrobial activity of different silver-containing products has been well-established and silver compounds are widely used in pharmaceutical preparations as effective antibacterial agents (Ruparelia et al. 2008). Today, increasing attention is being focused on metallic silver nanoparticles (NPs) for the protection of different products against microbial contamination. Although silver-containing organic compounds or silver ions have many medical applications as antimicrobial agents, these compounds cannot be

*Corresponding author. Email: Shahverd@sina.tums.ac.ir

widely used as disinfectant agents for the control of microbial pollution in open environments (e.g., waste water treatment plants) (Rosa-Gómez, Olgúin, and Alcántara 2008). Extensive application of silver NPs in wastewater treatment processes is not economical and silver NPs have also shown serious hazardous effects towards living organisms, which limits their wide applications in nature.

Composite magnetite NPs that contain two or more different nanoscale materials are of increasing interest in present day research because of their unique properties. These NPs have many applications, including use in nanoelectronic devices, molecular recognition, and biomedical applications (Wang, Wang, and Chen 2008). Magnetic nanoparticles (MNPs), for instance those composed of magnetite, have unique thermal, chemical, and magnetic properties that make them particularly well-suited for different applications (Prodan et al. 1999; Pham, Cao, and Sim 2008). Several procedures have been reported for the synthesis of different iron oxide NPs (Prodan et al. 1999) and their functionalization (Jolivet et al. 2010).

Moreover, the synthesis and antimicrobial potential of Fe_3O_4 -Ag core-shell MNPs or similar nanocomposites have been recently reported (Gong et al. 2007; Chudasama et al. 2009; Naik et al. 2011). In this strategy, Fe_3O_4 -Ag core-shell NPs can be easily removed from water using a magnetic field (Gong et al. 2007). The inhibitory effects of other silver compounds, especially silver chloride (AgCl), have also been reported (Choi et al. 2008). Insoluble AgCl has many applications in different fields of science and technology (Bates and Robinson 1980; Hares and Corning 1981). It is an effective antibacterial agent that is routinely used in wound dressings and healing products (Adams, Santschi, and Mellencamp 1999). In this study and for the first time, the preparation of Fe_3O_4 -AgCl nanocomposites was carried out by a precipitation method and the antibacterial activity of the nanocomposites was evaluated against *Escherichia coli*.

Experimental

Synthesis of Fe_3O_4 MNPs

The synthesis of Fe_3O_4 MNPs was carried out using a previously described method (Martínez-Mera et al. 2007). $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.487 g) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 g) were purchased from Merck (Darmstadt, Germany), precisely weighed and dissolved in 200 mL of deionized water. The mixture was then treated by the dropwise addition of 10 mL of NaOH solution (150 mmol L^{-1}) with mixing (200 rpm) under argon atmosphere using a laboratory magnetic stirrer (RHB2, IKA, Staufen, Germany). The final pH of the reaction mixture was adjusted to pH 12 with aqueous NaOH (150 mmol L^{-1}). The reaction mixture was then further incubated for 30 min at 80°C . The resulting black precipitate was separated by centrifugation ($8000 \times g$) (Tomy Seiko Co., Ltd., Tokyo, Japan). The separated MNPs were washed thrice with deionized water and used for the following Fe_3O_4 -AgCl nanocomposite preparation process.

Preparation of the Fe_3O_4 -AgCl nanocomposites

The MNP aqueous suspension (4% w/v) was prepared and mixed with 25 mL of NaCl solution (50 mmol L^{-1}). A volume of 25 mL of an aqueous AgNO_3 solution (50 mmol L^{-1}) was then added dropwise under strong mixing (300 rpm) using a laboratory magnetic stirrer. The resulting Fe_3O_4 -AgCl nanocomposites were separated from non-precipitated AgCl using an external magnet. The separated nanocomposites were re-suspended in

25 mL of deionized water and washed twice again with deionized water. Finally, the Fe₃O₄-AgCl nanocomposite particles were collected with an external magnet and used for the subsequent experiments.

Characterization of Fe₃O₄-AgCl nanocomposites

For transition electron microscopy (TEM), aqueous suspensions (2 mg mL⁻¹) of Fe₃O₄ MNPs and the magnetic Fe₃O₄-AgCl nanocomposites were dispersed by ultrasound at 130 W with an ultrasonic bath (Tecna 6, Techno-Gaz, Parma, Italy), and a drop of each suspension was placed on carbon-coated copper TEM grids and dried under an infrared lamp. TEM analysis was carried out using a TEM microscope (model EM 400, FEI-Philips/FEI, Eindhoven, the Netherlands) operated at an accelerating voltage at 80 kV. The particle size distribution patterns were evaluated by counting 500 NPs on each TEM micrograph. The elemental composition was determined by energy-dispersive X-ray spectroscopy (EDS) of a cluster of NPs. Further characterization was performed by X-ray diffraction (XRD) (Philips X'Pert Pro, Eindhoven, the Netherlands) to check for crystalline phases present in the samples.

Evaluating the chemical stability of the Fe₃O₄-AgCl nanocomposites

The chemical stability of the Fe₃O₄-AgCl nanocomposites was evaluated using a slurry (4 mg mL⁻¹) in deionized water (1000 mL). The suspension was transferred to a glass stopper Erlenmeyer flask and incubated under static condition in the dark at 25°C for 21 days. After quick shaking, aliquots of 150 mL were taken at day 0, 7, 14, and 21. The nanocomposites were separated using a laboratory centrifuge (20 min, 20,000 × g) (Tomy Seiko Co., Ltd., Tokyo, Japan) and dried under vacuum in the dark at room temperature. The silver content was determined as follows: 500 mg of nanocomposites were precisely weighed and added to 50 mL of concentrated HCl (37% v/v) (Merck, Darmstadt, Germany) in an Erlenmeyer flask. The flask was capped and incubated at room temperature for 24 h. The solution was filtered through a syringe filter (Albet, Spain), diluted, and subjected to flame atomic absorption spectroscopy (Varian 220, Varian-Agilent, Palo Alto, USA).

Antimicrobial susceptibility tests

Susceptibility tests were primarily studied using a standard serial agar dilution method (National Committee for Clinical Laboratory Standards 2000) in the Müller-Hinton Agar (MHA) medium, with an inoculum of approximately 10⁴ colony-forming units (CFU) mL⁻¹. *Escherichia coli* (ATCC 35218) was used as the test strain. The MHA was supplemented with serial concentrations of nanocomposites ranging from 1 to 16 mg mL⁻¹ (in two-fold steps). The data are reported as minimum inhibitory concentrations (MICs), the lowest concentration of Fe₃O₄-AgCl nanocomposites that inhibited visible growth after 24 h of incubation at 37°C.

The conventional time-kill course procedure was carried out by the following method. Nutrient broth culture media (Difco, USA) containing sub-MIC concentrations of Fe₃O₄-AgCl nanocomposites (0, 1, and 2 mg mL⁻¹) were prepared and inoculated with *E. coli* (2.62 × 10⁸ CFU mL⁻¹). All inoculated media were incubated in a shaker incubator (37°C, 150 rpm). The viable cell counts for each culture medium were monitored

at 10, 20, 30, 40, 60, and 80 min by the CFU quantification method (Putman, Burton, and Nahm 2005).

Antibacterial activity of recycled Fe₃O₄-AgCl nanocomposites against E. coli

Fe₃O₄-AgCl nanocomposites at a concentration of 2 mg mL⁻¹ in sterile distilled water were used. A fresh inoculum (3.91 × 10⁷ CFU mL⁻¹) was aseptically transferred to the nanocomposite liquid mixture (2 mg mL⁻¹) and inoculated flasks were incubated at 30°C for 1 h in a shaker incubator (100 rpm). Sterile distilled water without nanocomposites was inoculated, incubated, and used as control. Viable cell counts were determined by the CFU quantification method (Putman, Burton, and Nahm 2005). Using a magnet, washing the separated nanocomposites, and re-dispersing them in distilled water for the same test was repeated four times. The antibacterial killing rates for each recycling step were calculated using the following equation:

$$\text{Antibacterial killing rate (\%)} = \text{Viable cell number}_{\text{sample}} / \text{Viable cell number}_{\text{control}} \times 100.$$

Results and discussion

Characteristics of Fe₃O₄-AgCl nanocomposites

Representative TEM images of Fe₃O₄ MNPs and fabricated Fe₃O₄-AgCl nanocomposites are shown in Figure 1(A) and (B). The particle size histograms of the Fe₃O₄ NPs and prepared Fe₃O₄-AgCl nanocomposites shown in Figure 1(C) and (D) indicate that both particles varied in size from 2.5 to 20 nm, with an average size of 7.5 nm for Fe₃O₄ MNPs (Figure 1C) and 12.5 nm for Fe₃O₄-AgCl nanocomposites (Figure 1D). Analysis of the Fe₃O₄ NPs by EDS confirmed the presence of Fe and O element signals (Figure 2A). Semiquantitative analysis of EDS experiment shows about 77.5 atom-% Fe and 22.5 atom-% O, ascertaining that Fe₃O₄ NPs were present. EDS analysis of the synthesized Fe₃O₄-AgCl nanocomposites (Figure 2B) indicated the presence of iron and oxygen, with additional peaks contributed by Ag and Cl atoms. The copper and carbon peaks occurring in Figure 2(A) and (C) are due to the grid used for TEM imaging. XRD was used to determine whether crystalline phases were present in the nanocomposites (Figure 3A and B). The presence of both Fe₃O₄ and AgCl peaks in spectrum B verify the connection of MNPs with AgCl crystals. They were face-centered cubic and the dominant crystal planes were (111), (222), (311), (422), and (511) (Figure 3B).

Chemical stability of Fe₃O₄-AgCl nanocomposites

The Fe₃O₄-AgCl nanocomposites were suspended in distilled water for 21 days at room temperature. At different intervals, Fe₃O₄-AgCl magnetite nanocomposites were separated and the AgCl residues (% w/w) were determined by flame atomic absorption spectroscopy. Table 1 shows the percentage of AgCl (w/w) sampled at different times days and the respective loss in AgCl. Up to 40% was lost within 21 days. Within shorter periods of up to 5 h under mixing in a shaker (150 rpm), no change in AgCl was noted (data not shown).

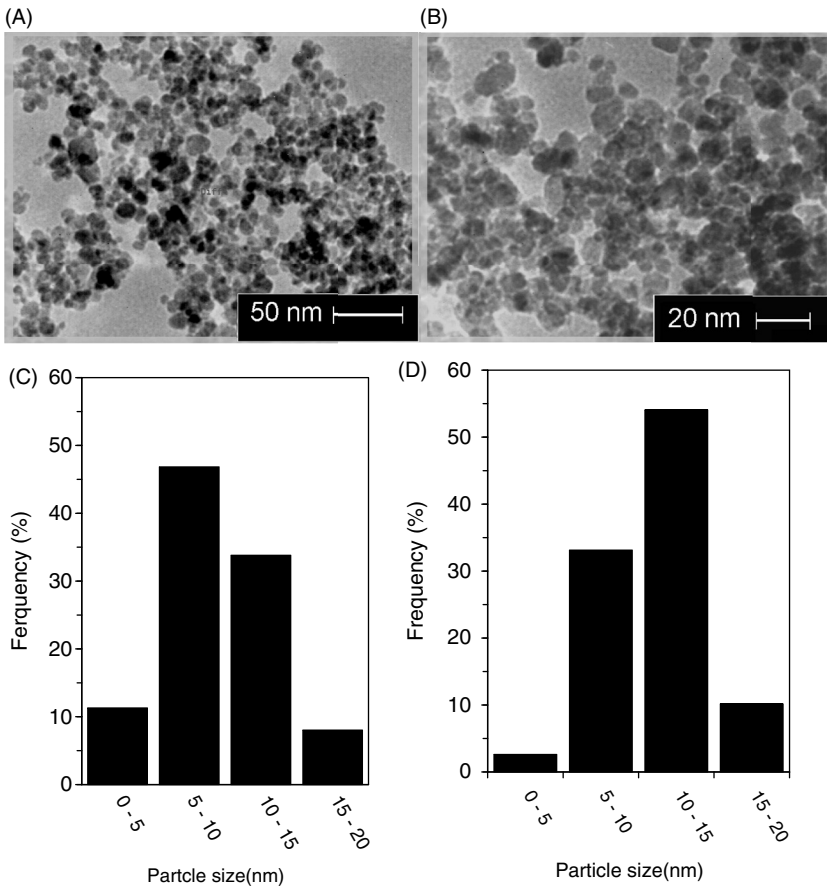


Figure 1. Representative TEM images of Fe₃O₄ NPs (A) and Fe₃O₄-AgCl nanocomposites (B). Lower illustrations show particle size distribution histograms of Fe₃O₄ NPs (C) and Fe₃O₄-AgCl nanocomposites (D).

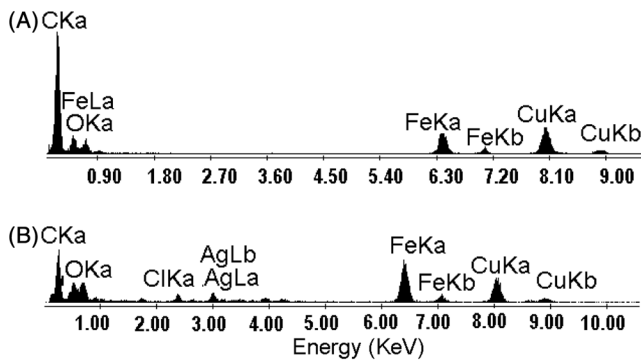


Figure 2. EDS spectra of magnetite Fe₃O₄ NPs (A) and fabricated Fe₃O₄-AgCl nanocomposites (B) using a precipitation process.

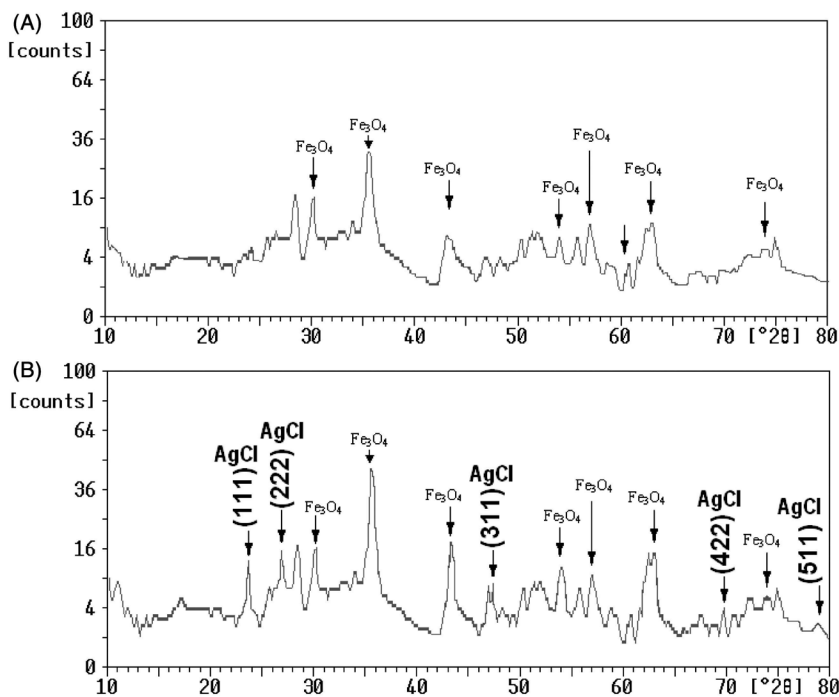


Figure 3. XRD pattern showing the presence of Fe_3O_4 (A) and $\text{Fe}_3\text{O}_4\text{-AgCl}$ (B) crystalline phases.

Table 1. Determination of silver in $\text{Fe}_3\text{O}_4\text{-AgCl}$ nanocomposites (1 g) before and after suspension in distilled water for 7, 14, and 21 days under static condition.

Time (day)	Silver content (mg g^{-1})	Remaining silver (%)	Silver lost (%)
0	16.4	100.0	–
7	13.8	84.1	15.9
14	12.0	73.1	26.9
21	5.6	58.5	41.5

Antibacterial properties of $\text{Fe}_3\text{O}_4\text{-AgCl}$ NPs

The MICs of Fe_3O_4 NPs and fabricated $\text{Fe}_3\text{O}_4\text{-AgCl}$ nanocomposites were determined for *E. coli* (ATCC 35218) by a conventional serial agar dilution. The MIC values of Fe_3O_4 NPs and the prepared $\text{Fe}_3\text{O}_4\text{-AgCl}$ nanocomposites were $> 16 \text{ mg mL}^{-1}$ and 4 mg mL^{-1} against the test strain, respectively. Comparison of the MIC values indicated that AgCl containing NPs could inhibit visible growth of the test strain at a lower concentration (4 mg mL^{-1}) and the nanocomposites were selected for further antibacterial assays. Higher concentrations of the uncoated magnetic Fe_3O_4 NPs showed a similar toxicity effect. The results of a time-kill course study carried out with $\text{Fe}_3\text{O}_4\text{-AgCl}$ nanocomposites against *E. coli* at sub-MIC concentrations are shown in Figure 4. Microbiological examination of

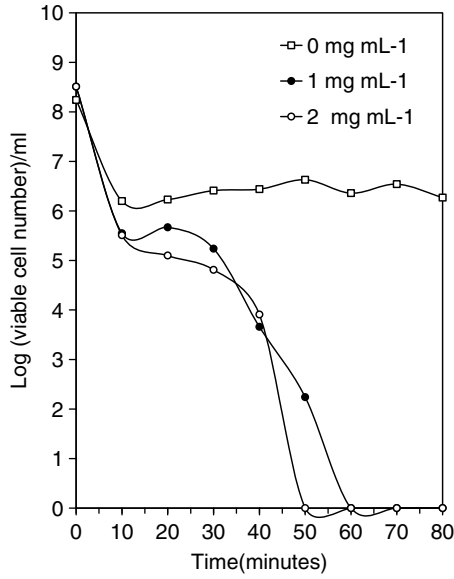


Figure 4. Time-kill kinetics of *E. coli* in the absence and presence of selected concentrations of magnetite Fe₃O₄-AgCl nanocomposites (1 and 2 mg mL⁻¹).

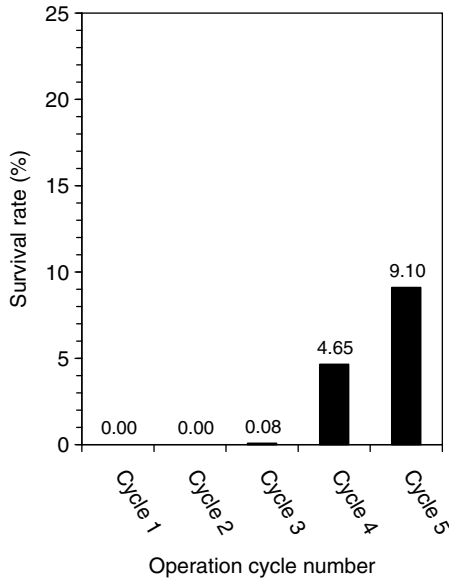


Figure 5. Antibacterial activity of Fe₃O₄-AgCl nanocomposites during different recycling processes.

the inoculated suspensions containing Fe₃O₄-AgCl nanocomposites (1 and 2 mg mL⁻¹) using the time-kill course method revealed no viable cells after 80 min. In contrast, we detected considerable numbers of viable cells (about 2 × 10⁸ CFU mL⁻¹) in sterile distilled water inoculated by the test strain (100 mL) and incubated under the same conditions.

The antibacterial activity of recycled Fe₃O₄-AgCl magnetic nanocomposites

Since fabricated Fe₃O₄-AgCl magnetic nanocomposites showed a good chemical stability over a short time period (5 h), we examined the antibacterial activity of recycled Fe₃O₄-AgCl magnetic nanocomposites (2 mg mL⁻¹) over four 60 min cycles of antibacterial treatment (Figure 5). As shown in Figure 5, the antibacterial efficiency of recycled Fe₃O₄-AgCl magnetic nanocomposites was constant during the first and second recycling process and no viable cells were detected in these stages. However, the antimicrobial efficiency of the recycled Fe₃O₄-AgCl magnetic nanocomposites began to decline in the third and fourth recycling tests.

Conclusion

In this study, Fe₃O₄-AgCl magnetic nanocomposites were synthesized using a precipitation method and tested for their capacity to kill *E. coli* cells suspending in water. To the best of our knowledge, this is the first report of the synthesis of Fe₃O₄-AgCl magnetic nanocomposites and their antibacterial efficiency against *E. coli*. These antibacterial nanocomposites can be used and recycled at least two times for successfully killing of *E. coli* (>99%). At the present time, the antibacterial effects of these nanocomposites against other test strains are not known, but merit further investigation.

Acknowledgments

This work was supported by a grant (No. 89-03-33-11116) from the Tehran University of Medical Sciences, Tehran, Iran. Also the partial support of Pharmaceutics Research Center, Kerman University of Medical Sciences (Kerman, Iran) is appreciated.

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