

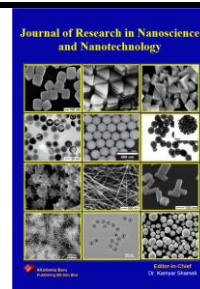
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Physiochemical synthesis of Silver/Kaolinite nanocomposites and study their antibacterial properties

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ABSTRACT

Silver nanocomposites (Ag NCs) were prepared by two methods, chemical and physical synthesis using sodium borohydride and gamma irradiation as a reducing tool. A one-step silver/kaolinite nanocomposite (Ag/Kln NCs) synthesis method has been developed successfully by irradiation technique at room temperature and under ambient pressure. The Ag/Kln NCs produced by the γ -irradiation technique is pure without chemical residues, has a good distribution with enhanced antibacterial properties, and environmentally friendly. The effects of various experimental parameters on the formation of NCs, such as the concentration of Ag⁺ and the irradiation dose, have been investigated. A study on antimicrobial susceptibility was undertaken to determine the antibacterial properties of Ag NCs in the presence of gram-positive and gram-negative bacteria. The susceptibility of the microorganisms to varying concentrations of Ag NCs synthesized via physical synthesis using gamma irradiation was compared to those synthesized chemically. Concentrations of Ag NCs used were 0.5, 1.0, 2.0, 5.0, and 10 % for chemical synthesis and irradiation doses used for physical synthesis were 7, 13, 20, 30, 40, 50, 65 and 80 kGy. Observation on well diffusion variant showed a significantly large zone of inhibition for physically synthesized NCs, (63 to 107% relative to control) which indicates high antimicrobial activity. Chemically synthesized NCs using the same experimental set up however showed a significantly smaller zone of inhibition. The Ag/Kln NCs in 20kGy showed higher antibacterial activity against *Enterococcus faecalis* and *Escherichia coli* as gram-positive and gram-negative bacteria. These suggest that Ag/Kln NCs can be employed as an effective bacteria inhibitor and can be applied in the medical field.

Keywords:

Silver nanoparticles; kaolinite; Gamma irradiation; Chemical synthesis, Gram positive, Negative bacteria.

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1. Introduction

Nanocomposites have been the subject of interest among researchers for its exceptional properties that differ from other conventional macroscopic materials [1]. One of the extensively studied nanocomposites is silver nanocomposites (Ag NCs) [2, 3] which have been widely used for medical and chemical applications due to their exceptional properties namely their antibacterial properties [4, 5], unique optical properties [6], electrical conductivities [7], oxidative catalysis and high resistance to oxidation and high thermal conductivity [8].

Many studies have been conducted in conjunction with the growth and increase of microbial resistance to multiple antibiotics to establish alternative bactericides [9] using metal NCs, like Ag-NCs, which exhibit a broad spectrum of antimicrobial activity against different species of microorganisms [10,17]. For the synthesis of Ag NCs, chemical reduction techniques have been widely explored because these techniques can be performed under simple and mild circumstances and can be used to synthesize Ag NCs on a big scale [18-20]. In the synthesis of Ag NCs by the reduction reaction, the decreasing capacity of a reductant has a significant role. The use of a powerful reductant, such as NaBH_4 , results in small, well distributed particles [3, 21-23].

For the synthesis of Ag NCs using silver salt as the initial materials, various production methods have been described; for example, chemical reduction, aerosol spraying technique, reverse micelle, lamellar liquid crystal, micro-emulsion, micelle, method of capping agent, and photochemical reduction (microwave, electron beam, ultraviolet (UV-irradiation and γ -irradiation). In comparison with traditional chemical and photochemical methods, the use of γ -irradiation in the preparation of Ag NCs has been shown to have a variety of highly advantageous properties [24-28]. Synthesis using γ -irradiation is simple, uncontaminated and uncomplicated while the Ag NCs produced are pure without impurities, fully reduced, harmless, and stable; the reductant is generated uniformly in the solution [29].

Ag NCs appears to agglomerate in aqueous system and this can be prevented with the presence of polymeric materials as stabilizing agents [8]. Therefore, polymers were added to help control and stabilize the particle formation, improve the dispersions and reduce the oxidation of the particle [30]. By using an inert carrier such as clay, the NCs produced are stable without complications [31]. Notably, kaolinite (Kln), a type of clay was found suitable as a support, carrier and stabilizers [32]. Kaolinite, is featured as a non-swelling clay [33], hence, is suitable and practical as solid supports for the preparation of Ag NCs and also to regulate the size of the particles as well.

In this study, silver nitrate (AgNO_3), a precursor salt is adsorbed on the surface of kaolin, which acts as a reactor and synthesized by both chemical and physical synthesis using sodium borohydride (NaBH_4) and γ -irradiation respectively to produce silver kaolinite nanocomposites (Ag/Kln NCs). When the mixture containing AgNO_3 solution and kaolinite was exposed to ionizing radiation, Ag^+ is reduced to Ag^0 by reactive radical produced from water radiolysis. Ag^0 coalesce to give metal NPs in presence of stabilizing agent kaolinite [34]. This work explored the antibacterial potential of Ag/Kln NCs by means of chemical synthesis using NaBH_4 as well as physical synthesis using γ -irradiation.

2. Materials and Methods

The silver precursor AgNO_3 (99%) and reduction agent NaBH_4 (99%) used as were obtained from QRéc (New Zealand). Meanwhile, the kaolin powder, used as solid supporter for the Ag NCs was purchased from R & M Chemicals (UK). In the preparation of all solutions, deionized water was used.

2.2 Sample Preparation

2.2.1 Synthesis of Ag-NPs using NaBH_4

Different concentration of AgNO_3 is prepared for the synthesis of Ag/Kln NCs. In 5 beakers with different volumes of distilled water, 0.04 g, 0.06 g, 0.16 g, 0.4 g and 0.8 g of AgNO_3 were simultaneously added into each respective beaker and protected from light for 1 hour. Then, with continuous stirring a constant amount of kaolin (5g) was added to each respective beaker. The suspensions were then stirred for 1 hour before adding freshly prepared NaBH_4 solutions, the reducing agent with the ratio $\text{AgNO}_3/\text{NaBH}_4$ (1:4). After the addition of the reducing agent, stirring was continued for another hour. The suspensions were centrifuged for 10 minutes, filtered using Buschner funnel and washed twice with distilled water to remove the silver ion residue, and dried in an oven. All the experiments will be conducted at room temperature.

2.2.2 Synthesis of Ag-NPs using γ -irradiation

Synthesis of Ag/Kln NCs, were carried out using five different doses of irradiation. 0.2M of AgNO_3 solution is prepared and added to the kaolin suspension prepared similar to the chemical synthesis. The samples are irradiated at 7, 13, 20, 40, 65 and 80kGy then centrifuged, filtered and washed similar to the chemical synthesis and finally dried in an oven. Gamma irradiations were carried out in 60-Co gamma chamber with dose rate of 4 kGy hr⁻¹ determined using Fricke dosimetry.

2.3 Antibacterial Assay by Well Diffusion Method

The pure cultures of *E. faecalis* (ATCC 29212) and *E. coli* (ATCC 25923) a Gram positive bacteria and Gram negative bacteria obtained from the Microbiology Laboratory of Universiti Teknologi Malaysia were cultured in Mueller-Hinton agar at 37°C for 24 hours. To maintain its viability, it was sub-cultured from time to time during the study period. The 20 μl of liquid Mueller-Hinton agar (pH 7.3 ± 0.2 at 25°C) was poured onto disposable sterile Petri dishes and allowed to solidify. Using 6 mm diameter a cork borer, a 6 mm holes were punched aseptically on the solidified agar. The agar plugs from each well was removed aseptically. Then 100 μl of the microbial culture suspension (CFU 107) was uniformly spread over the agar, using a disposable sterile rod and allowed to dry. Thereafter 50 μl of sample and control solutions were pipetted into respective wells and allowed to diffuse completely into agar before stacking the plates upside down and incubated at 37°C. The experiment was carried out in triplicate and after 24 hours the diameters of the zones of inhibition were measured. *Gentamicin* (10 mg/ml), sterile distilled water and kaolinite were used as control.

3. Results and Discussion

3.1 Characterisation of Ag/Kln NCs

For the synthesis using NaBH_4 , the colour of the solution instantly change to yellow when NaBH_4 was added indicating the formation of silver kaolinite nanocomposites (Ag/Kln NCs). As the concentration of AgNO_3 increased, so did the intensity of the colour turning darker to yellowish-brown. Meanwhile, for the synthesis using gamma irradiation, the colour of the suspension change from greyish to black according to the increasing irradiation dose. These colour change in all reaction flasks were the indication of the successful formation of Ag/Kln NCs. The colour intensity increased as a function of time due to the reduction of Ag^+ (**Fig. 1**). To verify the formation of Ag/Kln NCs and their stability, UV-Vis spectral analysis was performed.

3.2 U.V Analysis

The absorbance properties of various Ag/Kln NCs suspensions prepared by both chemical and gamma reduction were measured and the results are shown in Fig. 1. The silver surface plasmon resonance (SPR) bands were identified around 400 nm for both Ag/Kln NCs that can be assigned as SPR of Ag NCs [35, 36]. The UV-visible absorption spectra depends mainly on the morphology, structure and size of the NCs [36]. The symmetric shape of the plasmonic peak of Ag/Kln NSc imply the homogeneity of NCs in their size and shape.

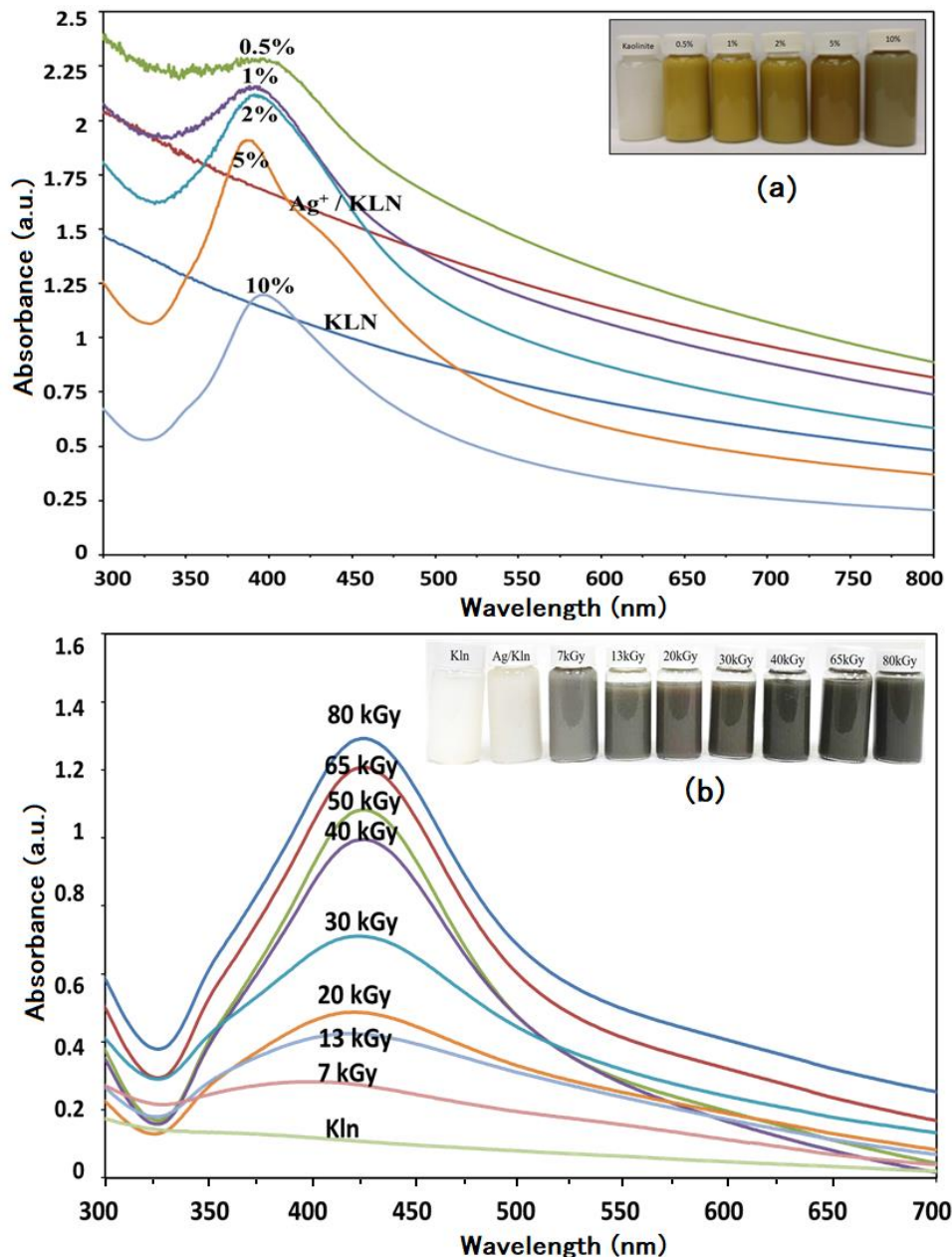


Fig. 1. The colour change due to the formation of Ag/Kln NCs using (a) chemical and (b) γ -irradiation synthesis with different percentages of Ag-NPs and γ -radiation dosage.

These absorption bands were presumably corresponding to AgNPs smaller than 10 nm. With creasing the concentration of the silver, the intensity of absorbance curves increased and they were shifted toward red wavelengths. The absorbance is directly proportional to the concentration of the

nanoparticles, indicating that the absorbance of maximum absorption wavelength (λ_{max}) increases with an increase in the number of nanoparticles. Based on this theory, nanoparticles with different sizes indicate different optical properties due to the difference in the surface plasmon resonance bands [35].

As for the gamma synthesis, as the dose of irradiation increases (7 to 80 kGy), so does the peak intensity of Ag/KIn NCs (**Fig. 1(b)**), due to an increase in the reduction of silver ions [34, 36]. The absorption peak of Ag NCs by chemical synthesis (**Fig. 1a**) indicates smaller size particles with narrow size distribution than those obtained by γ synthesis. This was further confirmed by TEM analysis of Ag/KIn NCs synthesized by γ -irradiation (**Fig. 2**).

3.3 TEM Analysis

The morphology of Ag/KIn NCs observed by TEM images, demonstrated well-formed spherical NCs with good dispersion (**Fig. 2**) indicating that KIn are good carriers for Ag NCs and their size are shown in the size distribution histograms based on TEM images beside each images. All Ag NCs distributed uniformly in the KIn matrix, which

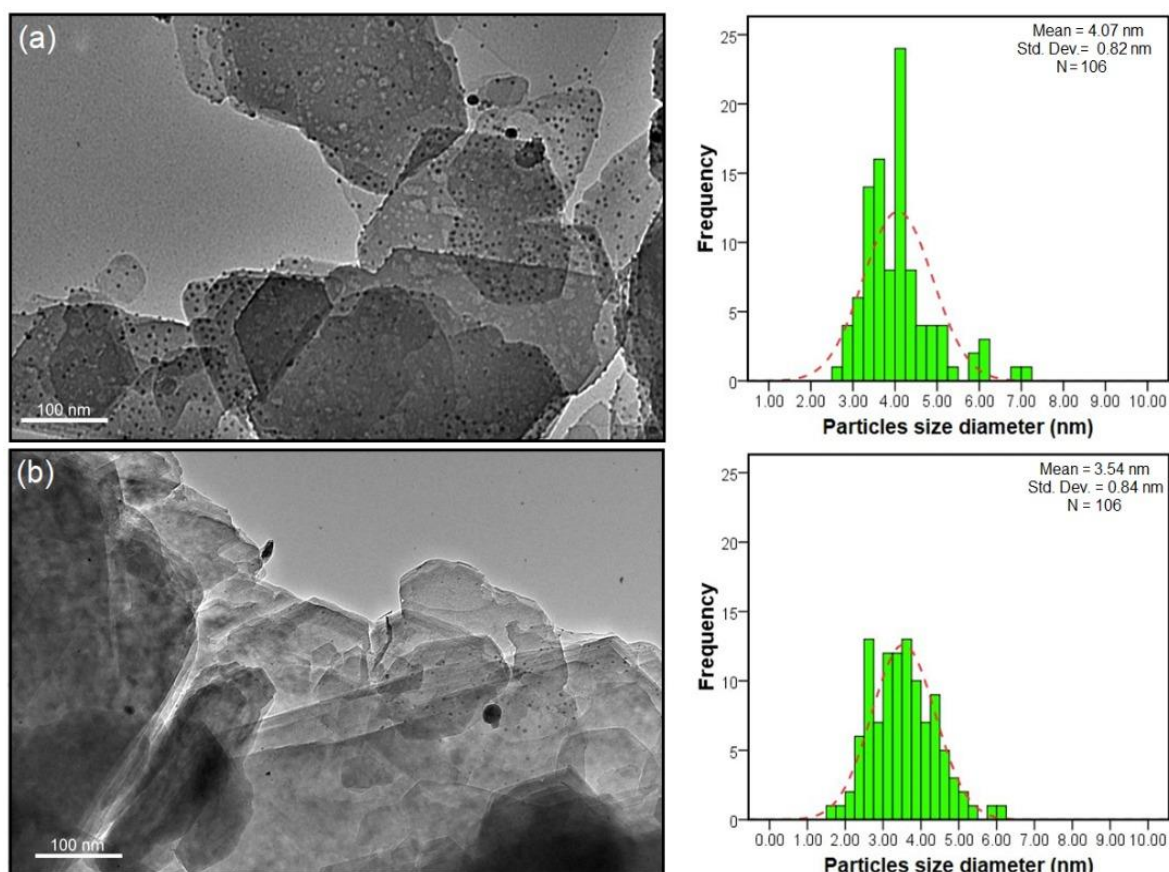


Fig. 2. TEM image of spherical Ag/KIn NCs prepared by (a) γ -irradiation and (b) NaBH_4 methods.

The average size of Ag/KIn NCs produced chemically was 0.95 – 16.01 nm, compared to γ -synthesized Ag/KIn NCs 4.28 – 16.13 nm, as shown in **Table 1** and in histogram (**Fig. 2**). The size of Ag/KIn NCs obtained increased consistently with the increasing of AgNO_3 concentration and γ -irradiation dose. Experimental findings have shown that with increasing precursor concentration of silver ions the size of NCs subsequently increased by γ -irradiation.

Table 1: Size of Ag/KIn NCs synthesized by chemical and γ -irradiation methods.

Chemically Synthesized		γ -synthesized	
Ag (wt.%)	Mean diameter (nm)	Dosage (kGy)	Mean diameter (nm)
0.5	0.95	7	4.28
1.0	1.26	13	4.94
2.0	2.75	20	9.19
5.0	15.82	30	9.91
10.0	16.01	40	10.01
		65	12.96
		80	16.13

3.3 Antibacterial evaluation

Overall, these results indicated that Ag/KIn NCs produced by both methods showed potential antimicrobial against both *E. coli* and *E. faecalis* bacterial. The control group consisting distilled water and KIn did not show any antibacterial activity against either gram-negative or gram-positive bacteria. Meanwhile the antibiotics, gentamicin were used as standard to determine the antimicrobial activity against bacterial pathogens.

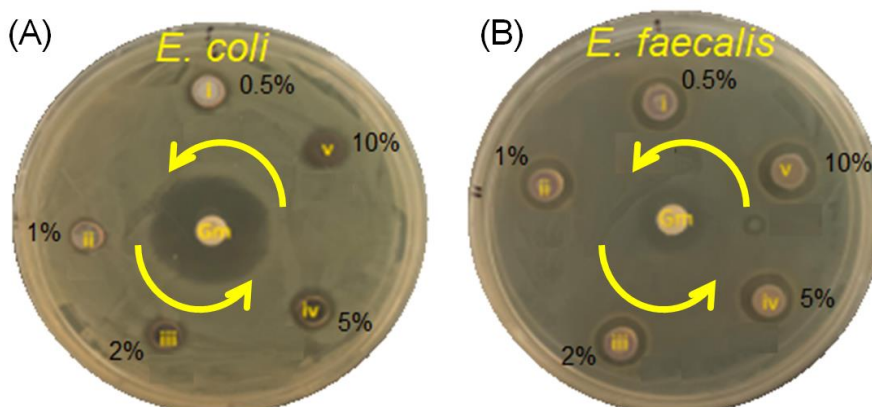


Fig. 3. Antibacterial activity of Ag/KIn NCs at 0.5, 1, 2, 5, and 10% against (A) *E. coli*, and (B) *E. faecalis* bacteria.

The zones of inhibition (corresponds to antibacterial activity) of Ag/KIn NCs synthesised chemically at different Ag concentrations namely 0.5, 1.0, 2, 5, and 10% wt., respectively was vaguely observed in both *E. faecalis* and *E. coli* [Fig. 3(A-B)]. However, the inhibition zone of Ag/KIn NCs synthesized using γ -irradiation showed a significant difference of $p < 0.01$ for inhibition zone, upon comparison to that of control (Gentamicin, 10 μ g/ml) suggesting that these NCs are effective antibacterial agents.

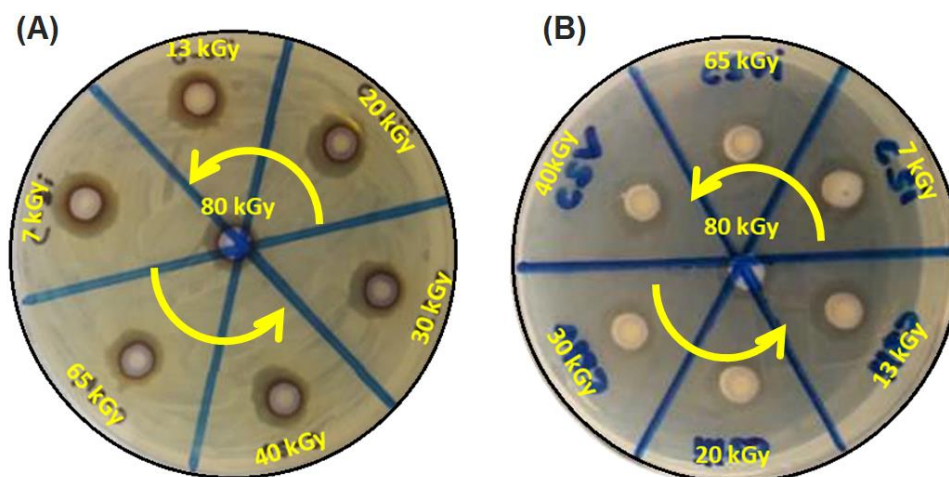


Fig. 4. Antibacterial activity of Ag/Kln synthesised using γ -irradiation at 7, 13, 20, 30, 40, 65, and 80 kGy against (A) *E. coli*, and (B) *E. faecalis*.

The mechanism responsible for the observed antibacterial activity of Ag/Kln NCs is the electrostatic attraction between the Ag and the negatively charged bacterial cells. Ag/Kln NCs is able to adsorb on the microorganism's cell wall and disrupts the integrity of the cell membrane thus causing a leakage of the cytoplasmic constituents and membrane damage, as well as enzyme inhibition of the organisms. Consequently, this leads to the death of the cells (12).

Table 2: Antibacterial activity of Ag/Kln NCs against *E. coli* and *E. faecalis* by well diffusion method measured in mm units.

Ag (%)	Diameter of Inhibition Zones Ag/Kln NCs		Dose (kGy)	Diameter of Inhibition Zones Ag/Kln NCs	
	Chemical Method			γ -Irradiation Method	
	<i>E. coli</i>	<i>E. faecalis</i>		<i>E. coli</i>	<i>E. faecalis</i>
0.5 %	9.00	6.70	7	10.61	11.00
1.0 %	8.00	6.70	13	10.67	11.04
2.0 %	8.70	7.00	20	12.50	11.68
5.0 %	9.30	7.00	30	12.00	11.57
10.0 %	8.30	7.00	40	11.50	11.25
			65	11.27	11.17
			80	11.43	11.03
Gm (10 μg/ml)	24.30	13.30		24.30	13.30

Note: Gentamicin was used as control.

Ag NCs with small diameter are said to have superior antimicrobial activity than those with larger diameter [37]. The Ag/Kln NCs synthesized using γ -irradiation demonstrated higher antibacterial activity for both *E. coli* and *E. faecalis* though, despite having larger size compared to NCs synthesized using chemical method. The highest inhibition was seen at 20 kGy (Table 2). Studies have shown that membrane permeability increase greatly by the released of Ag^+ in an aqueous solution and bacterial wall might be disrupted as a result of the extensive interaction between Ag^+ and DNA as well as phosphorous and sulphur containing proteins, leading to cell death [11]. It was reported that the reactivity of Ag NCs is enhanced with the smaller-sized (<10 nm), that apparently has superior penetration ability into bacteria, especially in Gram-negative [38]. However, results shown in Fig 5 & 6 indicated otherwise. Ag NCs produced by γ -irradiation synthesis demonstrated

a distinct inhibitory effect on the microbial growth of *E. coli* and *E. faecalis*, although they are larger in scale compared to Ag NCs produced chemically (Table 2). Similar results was obtained by previous studies where irradiation at 20 kGy enhanced antibacterial activity of composite films [39].

Kaolinite are non-swelling clay with strong hydrogen bonds and van der Waals forces between neighboring layers hindering intercalation of material [40]. The Ag⁺ in the Ag/Kln NCs produced via chemical synthesis could not be released and therefor displayed weak antimicrobial activities. Results showed by increasing the radiation dose, chain scission occur resulting in weakening of the strong hydrogen bonds and this will increase the release Ag⁺ ions into the media and increasing of the antimicrobial activity [41].

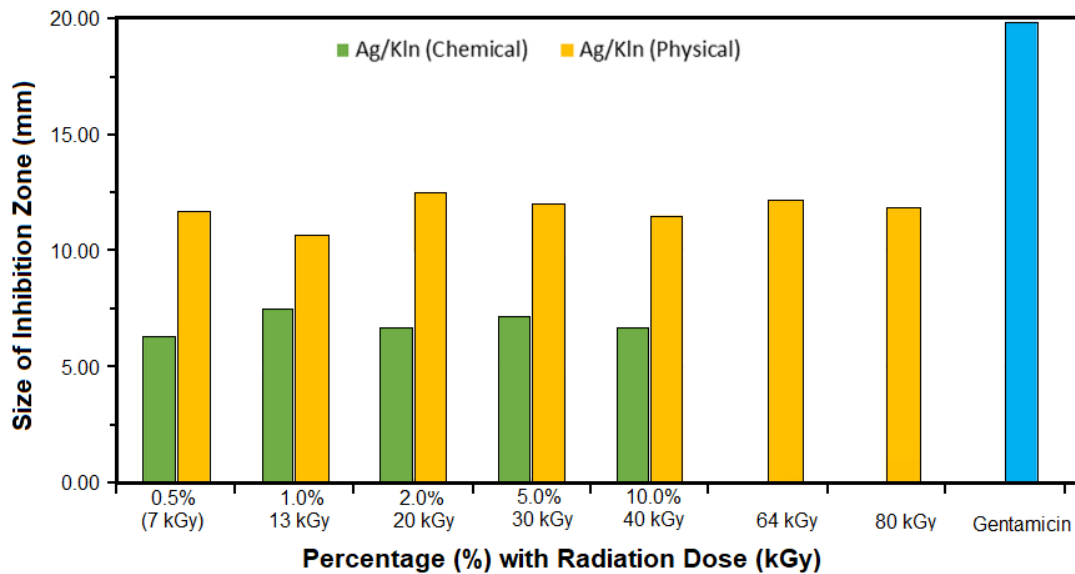


Fig. 5. Comparison between Ag/Kln NCs synthesised chemically and physically at different concentrations and irradiation doses on *E. coli* bacterium.

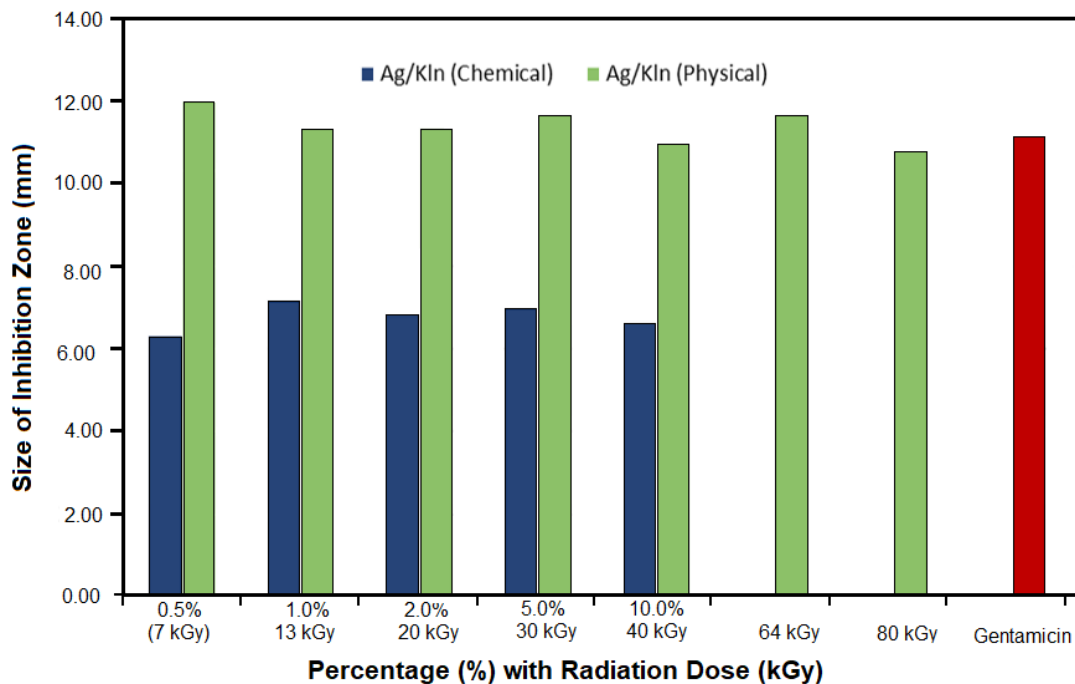


Fig. 6. Comparison between Ag/Kln NCs synthesised chemically and physically at different concentrations and irradiation doses on *E. faecalis* bacterium.

4. Conclusion

A simple and versatile approach was developed in this study for the synthesis of Ag NCs using gamma irradiation with different concentrations ranged between 0.02M and 1M. By using this method, samples of Ag NCs can be directly synthesized by irradiating the mixture consisting solution of kaolin and AgNO₃ at room temperature without any addition of reducer or other toxic, stabilizing and capping agents. In chemical synthesis of Ag NCs, sodium borohydride act as reducer agents whereas in γ -irradiation synthesis, no chemicals are involved making it an environmentally friendly procedure and the NCs produce are pure with no chemical residues. For chemical synthesis, a colour change of solutions from white to yellowish is observed due to excitation of surface Plasmon resonances (SPR) in metal NCs. The inhibition zone studies indicate that Ag/Kln NCs showed suitable antibacterial activity against *E. coli*, with a mean diameter that increased along with the Ag⁺ content of the system. For both NCs produced chemically and physically, showed the largest inhibition on *E. coli* (Gram negative) size followed by *E. faecalis* (Gram positive). Similar findings were also reported by previous studies. This may be due to Gram negative bacteria's thin cell wall, making it more vulnerable to the action of Ag/Kln compared to Gram positive bacteria (such as *E. faecalis*) that have thick peptidoglycan layers in their cell walls, making them more resistant to antibacterial agents. The highest inhibition of *E. coli* and *E. faecalis* was observed at 2-5% for Ag/Kln synthesized chemically. Ag/Kln NCs prepared using 20kGy demonstrated highest antimicrobial activity for both *E. Coli* and *E. faecalis* (Table 2). Gamma irradiation is a powerful tool, causing radiolysis and breaking of the strong Van der Waals bonding allowing the release of Ag⁺ and increasing the antibacterial potential of the gamma synthesised NPs. Gamma synthesized NPs (Figure 4 and 5), shows higher antimicrobial activity with a significant difference of $p < 0.005$. Chemical synthesis only produced 33-77% inhibition rates relative to control, whereas physical synthesis demonstrated 63-107% inhibition rates relative to control. This manifests future potential of these NPs against pathogenic microorganisms and creates more window of opportunity for research in this subject. Further studies for dose optimisation and toxicity of these materials are needed to enable its implication and use of these NPs to formulate novel antibacterial materials for various applications.

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