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ORIGINAL PAPER

Efficacy of Irradiated Bioactive Glass 45S5 on Attenuation of Microbial Growth and Eradication of Biofilm from AISI 316 L Discs: *In-vitro* Study

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Abstract Bacterial infection associated with medical implants remains a serious and costly drawback with both temporary and permanent consequences. Recently, some bioactive glasses have been found to show the antibacterial effect when interacting with bacteria. This study assessed the antibacterial activity of the bioactive glass (B.G.) based on 45S5 bioactive glass before and after treatment with gamma irradiation against multi-drug resistant microorganisms commonly involved in osteomyelitis. 45S5 glass which was synthesized by a melting technique with particle size <45 μm was characterized by optical absorption, optical band gap and Fourier Transform Infrared Spectroscopy (FTIR) before and after exposure to gamma irradiation. From the optical absorption, the optical band gap can be calculated. The antimicrobial activity of 45S5 glass irradiated at 25 and 50 kGy against some pathogenic strains was evaluated. The results revealed that irradiated 45S5 bioactive glass causes a reduction in the number of survivors of the tested strains rather than the non-irradiated one. The microbial counts decreased by increasing the pH. The results also indicated that the colony forming units (cfu) decreased by one log cycle in nutrient broth (NB) medium and three log cycles in simulated body fluid (SBF). Scanning electron microscopy showed cell shrinkage and membrane damage after exposure of the glass to irradiation. So, an alkaline pH and SBF media along with gamma irradiation are considered as the most preferable conditions affecting the antimicrobial activity of the irradiated 45S5 glass and have the ability to eradicate the biofilm produced by *P. aeruginosa* on AISI 316 L discs which are used in joint replacement.

Keywords Bioactive glass · Optical band gap, Infrared spectroscopy · Antimicrobial efficacy · Gamma irradiation · Biofilm formation

1 Introduction

The treatment of medical devices associated infections is usually performed using antibiotics. The spread of bacterial resistance in healthcare facilities is a worldwide problem; hence, the development of surfaces with low bacterial adhesion together with biocompatibility can represent a solution to prevent infections. Most researchers are now focusing on bone repair and replacement. Among a variety of materials for bone regeneration is the bioactive glass 45S5 which was discovered by Hench and coworkers [1, 2]. 45S5 bioactive glass promotes the bone growth and forms chemical bonds with the surrounding bone tissue and promotes the bone repair speedily and effectively. Meanwhile, it also has an excellent biocompatibility and self degradation [3, 4]. Bioactive glasses have a wide range of applications,

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such as bone grafts, scaffolds, coating materials, and are used for hypersensitivity treatment. One of the most important properties of bioactive glasses is their efficacy against most common Gram-positive and Gram-negative bacteria, which creates a bacteria- free environment while healing and regenerating the defect area [5].

Bellantone et al. [6] reported that the microbial adhesion to biomaterials was a fundamental step in bacterial infection, colonization and consequential biofilm formation. The biofilm structure comprises microbial cells and exo-polysaccharides providing an excellent environment to interfere with phagocytosis, to influence response to antibiotics and to function in later stages of such adhesion and subsequent failure of the surgery. Cells need their surface properties for pili, fimbria, fibrils and flagella in the process of adhesion [7]. So, efforts have been made in preventing the contamination on biomaterials during implantation. Combination of implant antibiotics may reduce the post operative infections, but complete infection eradication is still considered a challenging result. For better biocompatibility and applicability, the structure of B.G. 45S5 can be affected by different physical, chemical and biological modification, which can alter different aspects of the biomaterial such as degradation, hydrophilicity, bioactivity and sterility [8]. Gamma irradiation has been frequently used for sterilization of biomaterials affecting their structural and biological properties [9].

The aim of this study is to explain the impact of gamma irradiation on the structural and antimicrobial properties of 45S5 glass powder against some pathogenic strains commonly involved in osteomyelitis and the effectiveness of pH value and culture media on its antimicrobial activity were tested. The ability of the tested irradiated 45S5 glass to eradicate pseudomonal biofilm grown on AISI 316 L discs *in vitro* was tested.

2 Materials and Methods

2.1 Glass Preparation

The composition of the bioactive glass 45S5 is SiO_2 45%, Na_2O 24.5%, CaO 24.5%, P_2O_5 6%. The sample was prepared from high purity silica, calcium carbonate, sodium carbonate, and ammonium dihydrogen phosphate. Melting was done in a platinum 2% rhodium crucible in an electrically heated furnace regulated at 1400–1450 °C for about 2 hours. The glass was cast after complete homogeneity in a stainless steel mold 1×1×1 cm. The glass was properly annealed at a suitable temperature (450 °C) in a muffle furnace. The muffle furnace was left to cool at a rate of 30 °C/h down to room temperature and the glass was then crushed and dry ground in an agate mill to a powder of particle size <45 μm.

2.2 Preparation of Simulated Body Fluid (SBF)

Simulated body fluid (SBF) was prepared according to the procedure described by [10, 11].

2.3 Gamma-Irradiation Facility

The irradiation process was achieved using a ^{60}Co- gamma source (Russian Facility, Model Issledovatel) located at the National Centre for Radiation and Technology (NCRRT), Nasr City, Cairo, Egypt. The dose rate was 1.5 Gy/s (150 Rads/s) at a temperature of ∼30 °C. The different radiation doses exposure were 25, 50, 100 and 200 kGy.

2.4 X-Ray Diffraction Measurements (XRD)

The bioactive glass B.G. 45S5 was ground and the fine powder was examined by using the X-ray diffraction technique in order to identify the amorphous phases with a Philips diffractometer (PW 1390) adopting a Ni-filter and a Cu-target.

2.5 UV–Visible Measurements

Optical absorption measurements were performed on prepared glass samples exposed to UV–visible radiations in the range 200–800 nm with a CECIL UV–visible spectrophotometer. The obtained data have been used to calculate the optical band gap.

2.6 Fourier Transform Infrared Spectroscopy

The infrared absorption spectra of the bioactive glass 45S5 glass powder sample were analyzed at room temperature in the wave number range of 4000–400 cm^{-1} using Fourier transform infrared (JASCO FT/IR-4600). The prepared sample each of 2 mg was mixed with 200 mg KBr in an agate mortar and pressed into a pellet. These measurements were made for the prepared glass compositions before and after gamma irradiation.

3 Microbiological Studies

3.1 Microbial Strains

The antimicrobial activity of the 45S5 glass against six multidrug resistant (MDR) strains was investigated. They were chosen as they are commonly involved in osteomyelitis. Methicillin resistant *Staphylococcus aureus* (MRSA),

α-Streptococcus haemolytics (*α-Strept. haemolyticus*) and *β-Streptococcus haemolytics* (*β-Strept. haemolytics*), representing Gram +ve bacteria; *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*), representing Gram -ve bacteria and *Candida albicans* (*C. albicans*) representing yeast fungi. These MDR strains were obtained from the Radiation Microbiology Department (NCRRT).

3.2 Media

The tested organisms were cultured in standard laboratory culture media prepared according to the specifications of the manufacturers. Media utilized included Nutrient broth (NB), Nutrient agar (NA), Sabouraud agar (SA), Blood agar; Muller Hinton (MH) agar and broth were obtained from Oxoid (Oxoid. Comp., UK).

3.3 Substrate for Biofilm Formation

Discs with area 1.5 × 1.5 cm made from the AISI 316 L alloy usually used for joint replacement.

3.4 Effect of Gamma Irradiation on the Antimicrobial Activity of the Tested 45S5 Glass Powder

This test was carried out according to Allan and Newman [12] as follows: fresh 18 h cultures of the tested strains in NB (10^6 cfu/ml) were dispensed in 50 µl volumes into Epindorf tubes containing 0.05 g of irradiated (25 and 50 kGy) and non-irradiated B.G. 45S5, tubes without 45S5 glass were used as a control. The cultures were then incubated aerobically for 3 h at 37 °C. Following incubation, 950 µl of sterile phosphate buffered saline (PBS) was added. The samples were then vortexed for 1 min. and viable counts were performed by serial dilution in PBS and planting on nutrient agar plates. Following incubation at 37 °C for 24 h the resultant colonies were counted. All the experiments were carried out in triplicate.

3.5 Effect of Gamma Irradiation on the Antimicrobial Activity of 45S5 Glass Supernatants

Sterile NB (10 ml) was added to universals contained 5 g of 45S5 glass (irradiated and non-irradiated), then they were mixed on a rotator for 3 h at 37 °C. After time, 950 µl of the supernatants was added to tubes containing 50 µl of an overnight culture of tested strains (10^6 cfu/ml). The cultures were then incubated 1 h at 37 °C, serially diluted in NB, plated on NA plates and incubated aerobically at 37 °C for 24 h. The obtained colonies were counted and expressed as colony- forming units (cfu/ml.). The experiment was carried out in triplicate [12].

3.6 Effect of pH on the Antimicrobial Activity of Irradiated 45S5 Glass

The irradiated 45S5 glass supernatants were prepared as above. ANB set of test tubes with pH 9.8 and another with pH 6.5 were prepared. Tubes of NB without irradiated B.G. 45S5 were used as a negative control. Then 950 µl from each was added to another tube containing 50 µl of an overnight culture of the applied strains (10^6 cfu/ ml). Following incubation at 37 °C for 1 h, they were serially diluted in saline, plated on NA plates and incubated at 37 °C for 24 h.

3.7 Effect of Culture Media on the Antimicrobial Activity of Irradiated 45S5 Glass

Bioactive glasses 45S5 glass supernatant was produced as above using sterile NB or SBF as prepared in Section 2.2. Then 950 µl was added to an Epindorf tube containing 50 µl of an overnight culture of testing strains (10^6cfu/ml). The cultures were incubated at 37 °C for 1 h, serially diluted, plated onto NA plates and incubated at 37 °C for 24 h. The resultant colonies were counted.

3.8 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES)

The release of ions from (50 mg) irradiated 45S5 glass at 25 kGy and non-irradiated during 24 h contact with NB was measured by an inductively coupled plasma-optical emission spectrophotometer (ICP-OES) (Ciros; Spectro Analytical Instrument, Germany). An inductively Coupled Plasma Optical Emission Spectrometer Type: OPTIMA 3300 DV Manufacturer: Perkin-Elmer Ltd. The ICP-MS technique was used to analyze the trace element contents (Si, Ca, Na and P) of the 45S5 glass immersed in NB, after vortexing and incubation for 24 h, the samples were suspended in 5 ml distilled water, mixed and filtered. The determination was performed in duplicate.

3.9 Biofilm Formation

Biofilm formation was quantified for the tested strains in microtitre plates using the spectrophotometric method described by Rachid et al. [13]. Each strain was assayed in triplicate.

3.10 Anti-Biofilm Formation Activity of Irradiated and Non-Irradiated 45S5 Glass

The anti-biofilm activity of irradiated and non-irradiated 45S5 glass was evaluated through testing its ability to

eradicate the formed biofilms of the selected strain on AISI 316 discs by a spectro-photometric method according to Coraca-Huber et al. [14] as follows: Discs of AISI 316L which is commonly employed for joint replacement implants for its good mechanical properties and low costs, Antunes et al. and Batory et al. [15, 16] used it as a substrate for bacterial biofilm. Discs were washed, autoclaved at 121 °C and immersed in 48-well plates containing 1 ml of (2×10^5 cfu/ml) of the tested strain at 37 °C for 24 h on a rocking table (12 cycle/min.). The discs were washed three times with distilled water to remove the planktonic cells and transformed to another 48-well plate. In each well, 500 mg of 45S5 glass (irradiated at 25 kGy and non-irradiated) were placed. Glass beads (500 mg) were used as a control. Then 500 µl of Muller Hinton broth (MHB) was added to each well. The plates were incubated at 37 °C for 24 h, the discs were removed, washed three times with PBS and transferred to a 15 ml Falcon tube containing 2 ml of MHB. The tubes were sonicated for 1 min at 100% intensity for disruption of the biofilms. Then 20 µl of the sonicated fluid was added to MHA plates and incubated at 37 °C for 24 h. The resultant colonies were counted for the biofilm recovery verification. All the experiments were carried out in triplicates.

3.11 Scanning Electron Microscopy

In order to find out and elucidate the changes in the morphology of bacterial cells incubated with irradiated 45S5 glass, scanning electron microscopic (SEM) acquisitions were conducted on bacteria after 3 h incubation with 500 mg/ml of 45S5 glass. Cells of *P. aeruginosa* before and after treatment were fixed overnight with 2.5% glutaraldehyde, buffered with 0.1 M sodium phosphate buffer (pH 7.2) for 1 h at room temperature and then washed four times in sodium phosphate buffer, and postfixed in 1% osmium tetroxide in the same buffer for 1 h and washed in the same buffer again. They were then dehydrated in a graded alcohol series (30, 50, 70, 90, and 100%). All samples were subsequently dried overnight, gold coated and were examined in the JEOL- JSM -5400 scanning electron microscope (Japan).

4 Result

4.1 Characterization of Prepared 45S5 Glass

Figure 1 shows the characteristic X-ray diffraction pattern of 45S5 glass. It contains a broad hump and no shape line is observed which suggested that the prepared B.G. 45S5 sample confirms the amorphous (glassy) nature.

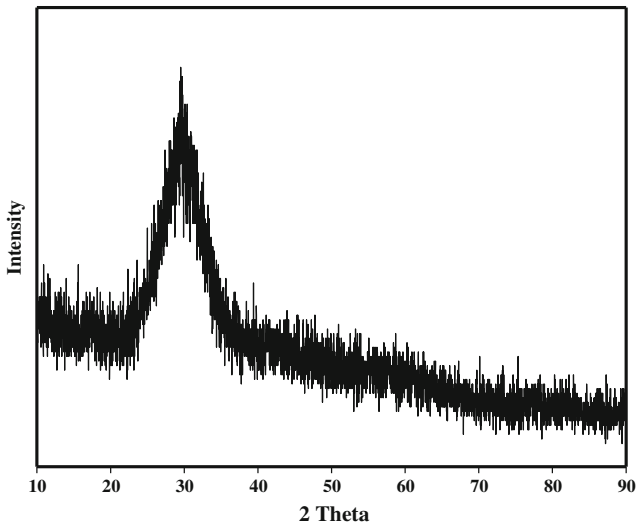

Fig. 1 XRD patterns of bioactive glass

4.2 Optical Absorption Before and After Gamma Irradiation

Figure 2 illustrates the UV-visible absorption spectrum of 45S5 glass. The spectrum was recorded from 200–800 nm, it reveals a strong UV absorption band around 236 nm, and no visible absorption is observed.

The absorption region shows a prominent induced band at 236 nm which shows an increase in the intensity on progressive gamma irradiation and also, the new visible band appearing at 500 nm was induced at 25, 50, 100 and 200 kGy Fig. 2.

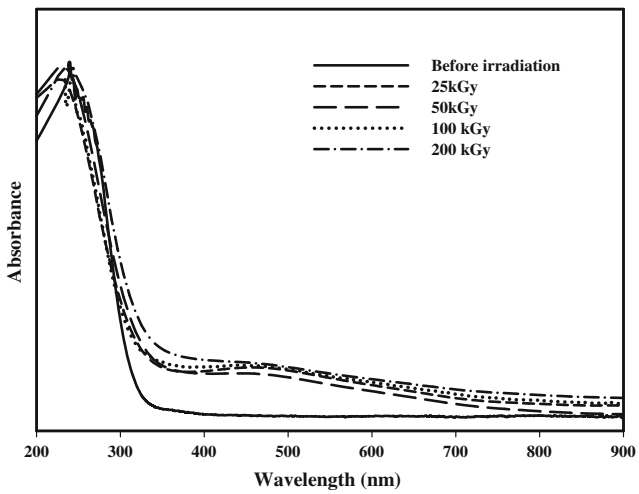

Fig. 2 Optical absorption spectra of bioactive glass before and after different doses of gamma irradiation

4.3 Optical Energy Gap Before and After Gamma Irradiation

The study of optical absorption is a useful method for the investigation of optically induced transitions and for the provision of information about the band structure and energy gap of non-crystalline materials, the optical transition is preferred to indirect transition due to lack of translation symmetry where the wave vector (momentum) is not defined. The energy gap can be calculated by Eq. 1 as given by Mott and Davis [17].

$$(\alpha h\nu)^{1/2} = B(h\nu - E_g) \quad (1)$$

Where hν is the incident photon energy, B is the band tailing parameter constant, Eg is the optical band gap energy, and a is the absorption coefficient determined by the formula ($\alpha = 2.303 \times$ Absorbance/glass thickness) in units of cm^{-1}. Figure 3 shows the variation of the optical transition $(\alpha h\nu)^{1/2}$ vs. the incident photon energy for the studied glasses. The optical band gap value can be determined from extrapolating the linear region of the curve to the hν axis where $(\alpha h\nu)^{1/2} = 0$ and the values are listed in Table 1 for all samples before and after irradiation.

4.4 FTIR Spectra

IR spectroscopy is a sensitive technique to the local structure of silicate glasses, which allows one to determine changes of the Si-O-Si vibrational modes, the breakage of Si-O-Si bonds and the formation of Si-O-NBO groups, which play an important role in the biological response at the interface of the bioactive materials when exposed to body fluids. This is due to the presence of SiO_2 as the major component (45%); such IR spectra are known to be composed of Si-O-Si stretching and bending modes beside the modes due to non-bridging oxygens. Figure 4 shows IR spectra of prepared bioactive glass before and after irradiation at 25, 50, 100 and 200 kGy. By comparing the IR spectra of the samples before and after irradiation of 45S5 glass it is clear that there is a slight increase in the region 900–1200 cm^{-1}.

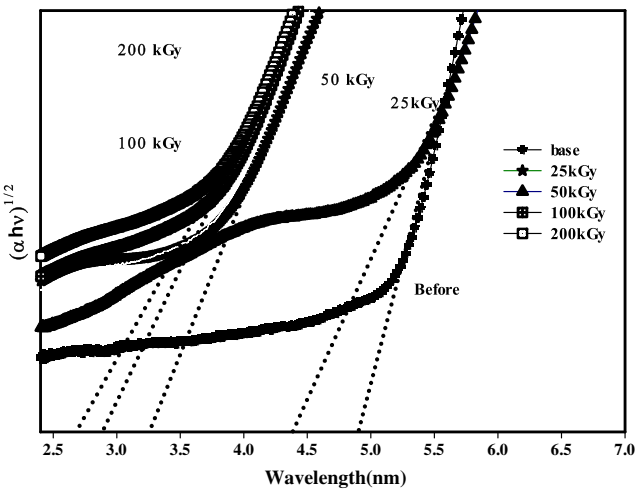

Fig. 3 Indirect optical band gap determination before and after different doses of gamma irradiation 25, 50, 100 and 200 kGy

Table 1 Indirect energy band gap of 45S5 glass

Dose (kGy)	Band energy gap (eV) for Bioglass Indirect
0	4.932
25	4.387
50	3.267
100	2.897
200	2.692

5 Application of Microbiological Studies

The 45S5 glass powder was submitted to 25 and 50 kGy of gamma irradiation and its activity against the applied strains, compared to non-irradiated 45S5 glass, was evaluated. Figure 5 shows that there was a notable antimicrobial activity of non-irradiated 45S5 glass against all the tested strains with % reduction ranging from 35.7 to 65.0%. While after exposure of 45S5 glass to 25 and 50 kGy gamma irradiation, a significant reduction ($p < 0.05$) in microbial counts for the tested pathogens ranging from 47.8 to 79.2% and from 50.1 to 80.9%, respectively, was observed.

The antimicrobial activity of 45S5 glass powder (irradiated and non-irradiated) was tested by leaving it in contact with an aqueous solution to activate the release of ions. Results in Fig. 6 reveal that direct contact between non-irradiated 45S5 glass and bacterial cells causes a reduction in the cells viability of all tested strains ranging from (40.5 to 67.2%). The treatment of cultures with irradiated 45S5 glass at 25 and 50 kGy caused a significant % reduction from 58.7 to 85.9% and from 60.6 to 89.0% respectively. At the same time, there were no significant differences ($p \geq 0.05$) between the antimicrobial efficacy of 25 and 50 kGy. So 25 kGy is chosen for further studies.

The impact of irradiated 45S5 glass at two different pH values of the medium (6.5 and 9.8) was studied against the tested strains. The results recorded in Fig. 7 demonstrated that pH 9.8 significantly affected the growth of the tested

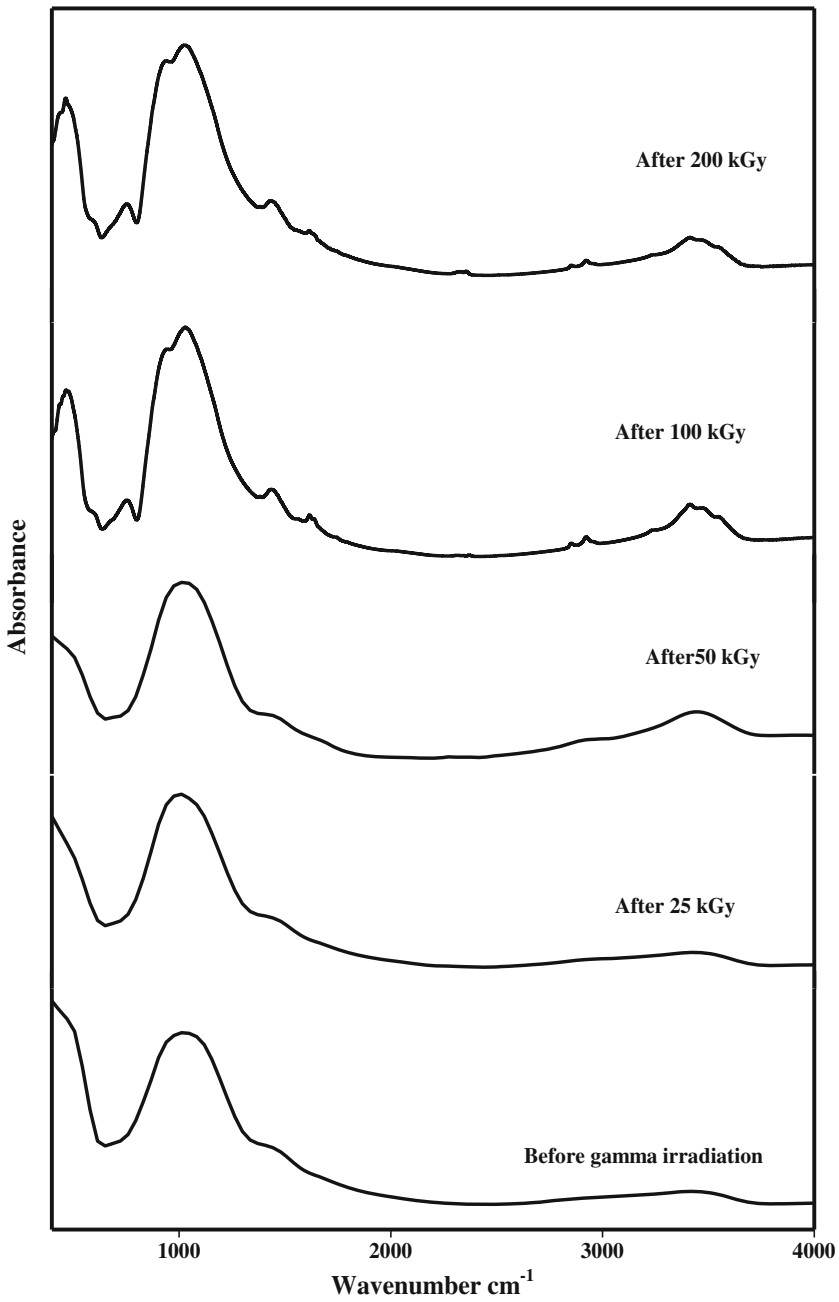

Fig. 4 Infrared absorption spectra of bioactive glass before and after different doses of gamma irradiation

strains with % reduction ranging from (75.7 to 90.2%), while pH 6.5 caused % reduction ranging from (35.8 to 51.3%). Also, the effect of culture media on the activity of the tested irradiated 45S5 glass (25kGy) was studied, and the results in Fig. 8 indicate that SBF clearly affected the growth of all tested strains.

There is a significant increase in ion release concentrations before and after treatment of 45S5 glass at 25 kGy Fig. 9. Concentration of Si ions was the highest over the tested ions (455.6 ppm) after gamma irradiation. While a pronounced increase in the concentration of Na, P and Ca ions was noticed after the exposure to gamma rays.

All the applied pathogens were evaluated quantitatively for their ability to produce biofilm. It was found that *P. aeruginosa*, *E. coli* and *MRSA* had an optical density (OD_{595}) of 0.962, 0.654 and 0.607 respectively, so they were quantified as strong biofilm producers,while *β-Strept. haemolyticus*, *α-Strept. haemolyticus* and *C. albicans* having an OD_{595} between 0.2 and 0.4 were classified as moderate biofilm producers. As *P. aeruginosa* was the most potent biofilm producer organism, it was chosen to study its adhesion on AISI 316L discs in the presence of irradiated and non-irradiated 45S5 glass on NB or SBF as the culture medium. It was found that non-irradiated 45S5 glass could

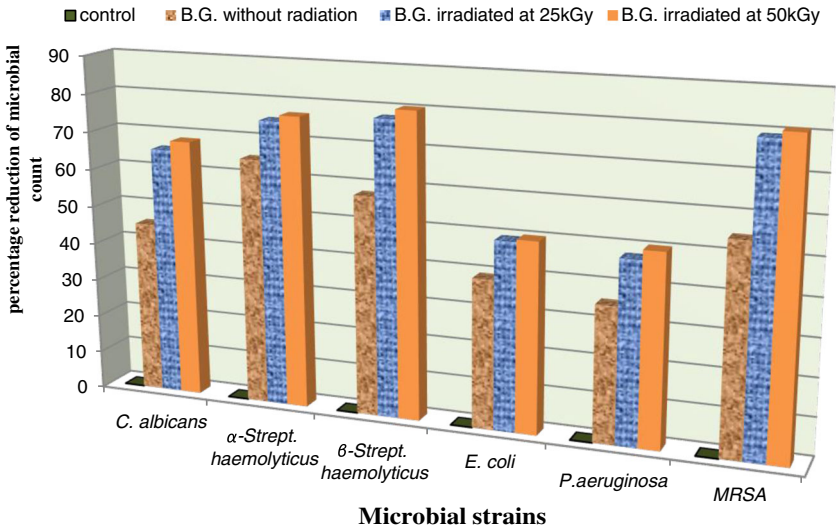

Fig. 5 Antimicrobial activity of irradiated and non- irradiated 45S5 glass against some pathogenic strains

suppress the biofilm formation of the tested strain grown on NB and SBF by one and three log cycles, respectively, while irradiated 45S5 glass causes a suppression rate of two log cycles with the NB medium. Meanwhile, 100 cfu/ml was the count for irradiated 45S5 glass on SBF culture medium, Fig. 10.

Morphology of normal *P. aeruginosa* cells and treated with irradiated 45S5 glass were observed using SEM; the results showed a change in the morphology of the treated bacterial cell after 3 h of incubation. Shrinkage and consequent distortion of the cell membrane was observed, Fig. 11b. Also, the treated cells with irradiated 45S5 glass showed shortening with an increase in their compactness. In contrast, untreated cells displayed a smooth and intact surface, Fig. 11a.

6 Discussion

Preventing and/or limiting bacteria colonization and contamination when a biomaterial is introduced into the body, is the main concern of a number of studies over the recent years.

The use of combined drug-materials, and especially biomaterials showing antimicrobial activity, is bound up with a lower probability of surgery failure by infections and an increase of application areas. Among biomaterials, bioactive glasses have exhibited antibacterial activity whose characterization before and after exposure to gamma irradiation has been established.

6.1 Optical Absorption of B.G.45S5 Before and After Gamma Irradiation

The ultraviolet, visible spectroscopy (UV-Vis) and calculated band energy gap can be useful tools to provide direct information about the physico-chemical changes in 45S5 glass before and after gamma irradiation. These measurements and calculations can predict the changes which occur after immersion in SBF solution and these changes may be attributed to the ion exchange of some cations from the 45S5 glass.

The observed UV absorption band at 236 nm can be assumed to originate from the presence of trace unavoidable iron impurities species Fe^{3+} and Fe^{2+} in the raw materials used to prepare the studied glass [18, 19]. Iron can exist in the 2+ and 3+ states in glasses, depending upon the conditions of preparation. The two iron states have strong charge transfer bands with very different absorption coefficients, being more than one order of magnitude higher for Fe^{3+} than for Fe^{2+}. The Fe^{2+}/Fe^{3+} redox ratio even in the ppm level has an extremely high influence on the UV absorption of high-purity glasses [20]. Also, the broadness of the UV absorption band extending from 200 to 300 nm in the present case is attributed to the possible presence of more than one site of both iron species (Fe^{2+} and Fe^{3+} ions) [21]. Particularly, the intense charge-transfer absorption near 236 nm in glass is related to Fe^{3+} ions. The divalent metal ions with s^2 configuration are known to absorb strongly in the ultraviolet and yield an absorption band center at about 236 nm. This result is supported by recent investigations done by several authors in borosilicate, alkali borate, bioglass, cabal and various commercial glasses in several publications [22, 23]. They have concluded that these glasses exhibit characteristic charge transfer ultraviolet absorption bands because of the presence of transition metal ions (e.g. Fe^{3+}, Cr^{6+}, etc.) even if present in the ppm level. Gamma rays are likely to produce the UV and visible defects which are related to both the effect of irradiation on the silicate glass, as well as due to extrinsic trace iron impurities. These induced defects which are generated from the host glass are implicitly located in the same positions which are covered by the

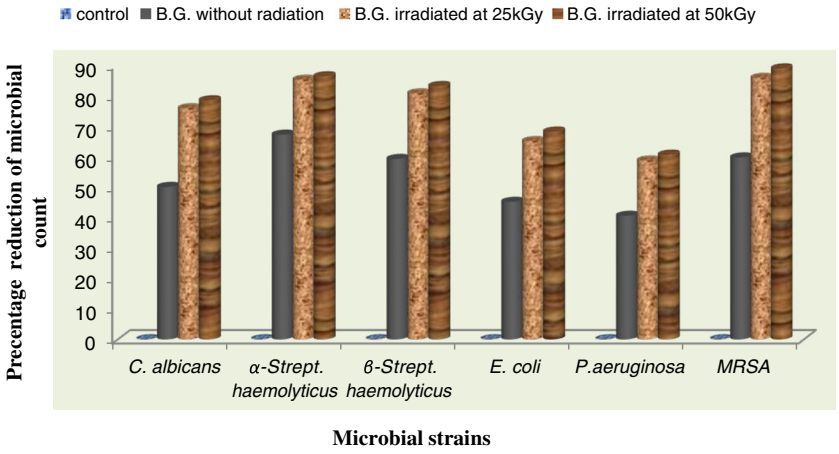

Fig. 6 Antimicrobial activity of supernatants of irradiated and non- irradiated 45S5 glass against the tested strains

absorption bands due to iron impurities [24]. In the light of this discussion it can be concluded that a presence of trace unavoidable iron impurities in irradiated 45S5 glass did not affect its potential role against bacterial cells.

6.2 Effect of Gamma Irradiation on Optical Band Gap of 45S5 Glass

The interaction of glass with nuclear radiations can cause the breaking up of three dimensional networks leading to disturbing bridging oxygens (BO) or creating two non bridging oxygen (NBOs) per Na_2O molecule with Na^+ ions occupying the sites in the SiO_2 network. Principally, the negative charges on NBOs have a larger magnitude as compared to that of bridging oxygen. Increase of the ionicity of oxygens ions by transforming them from bridging oxygen ions to non bridging oxygen ions raises the top of the valence band resulting in the reduction of band gap energy. Consequently, the UV absorption occurs at lower photon energies as the oxygen atoms become NBOs as observed by Moncke et al. [25]. From the previous discussion, it can be predicted that the 45S5 glass network become weaker, so when immersed in SBF the rate of ion exchange increases after irradiation and the ions which are released cause an increase in the pH of the solution that might play an important role against the bacterial cell.

6.3 FTIR Spectra

FTIR spectra of 45S5 glass before irradiation confirmed that the bands appearing at 510 cm^{-1} and around 745 cm^{-1} were attributed to the Si-O bending mode. Moreover, the vibration mode of Si-O non-bridging oxygen appeared between 895 and 970 cm^{-1}. While the band located in the range 1000–1200 cm^{-1} is due to the Si-O stretching mode, the band at 1460 cm^{-1} is related to the carbonate group. The

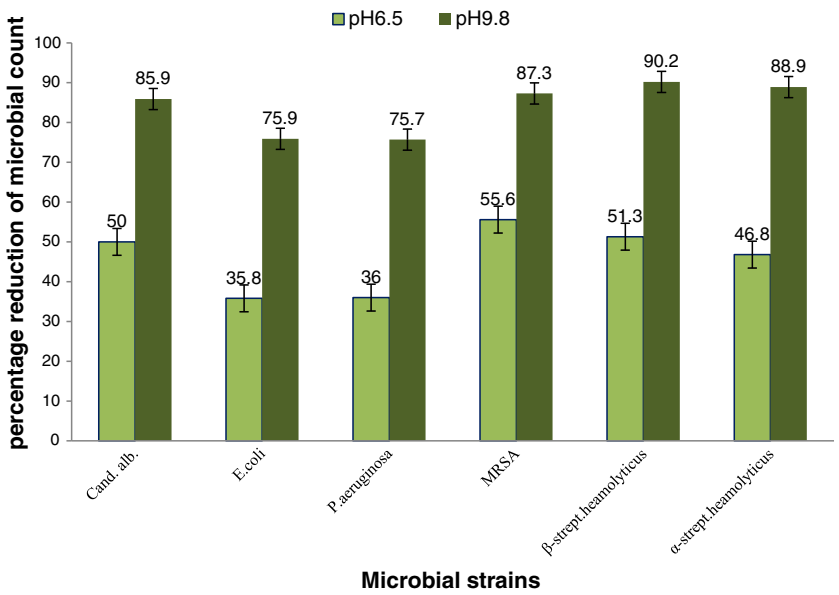

Fig. 7 Effect of different pHs on antimicrobial activity of irradiated 45S5 glass against the tested strains

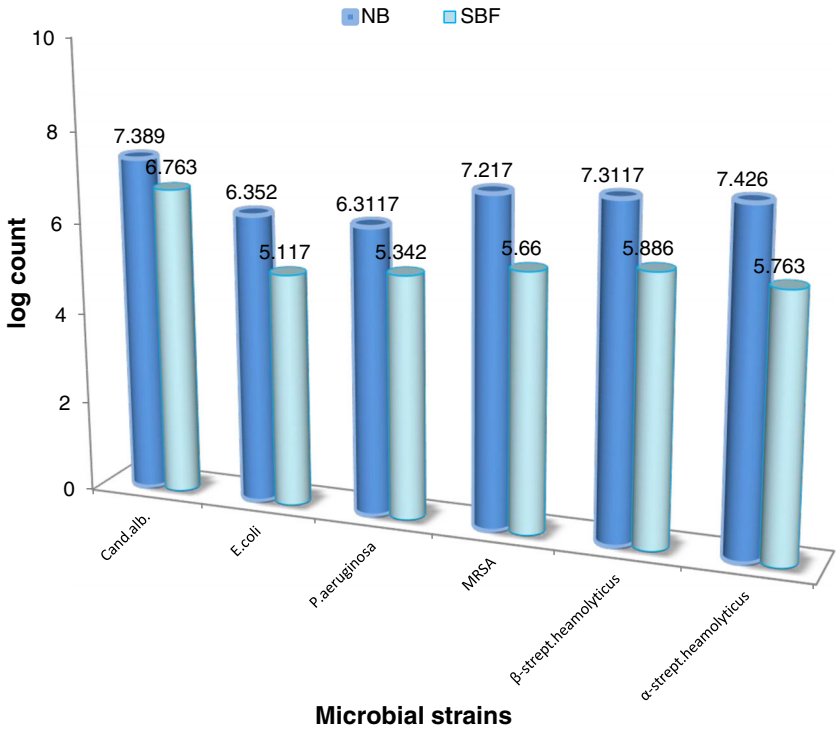

Fig. 8 Effect of culture medium on antimicrobial activity of irradiated 45S5 glass against tested strains

band around 1640 cm^{-1} is related to molecular water, and the band at 3450 cm^{-1} corresponds to different modes of water, OH or silanol groups [26]. FTIR spectra of BG after irradiation confirm that the bands reflect the change taking place in the glass structure after interaction with gamma irradiation. It can be assumed that gamma irradiation causes structural disorder including displacement, electron rearrangement, radiolysis and this leads to changes in the bond position and/or the bond angle of the building units. The slight change in the intensity also affects the high surface area of the silica structures. The radiation induced damages generated in glasses depend on the dose of radiation, the type and composition of the glasses, and the intrinsic defects within them. The radiation damage processes which take place in glasses generally occur in one of three basic processes [27]: (1) radiolysis, (2) displacement, and (3) electron rearrangement. However, after irradiation doses at 50,100, 200 kGy, the network forming units (SiO_2) remain unchanged. In other words all the main vibrational bands remain almost unchanged in their numbers and positions. These results indicate that the base silicate has marked resistance against gamma irradiation after 25 kGy. The same assumption, previously introduced by several authors [28–30] and adopted by El-Batal et al. [31], that structure of silicate groups are somewhat resistant to gamma irradiation and the infrared spectra is seen to be quite pronounced with

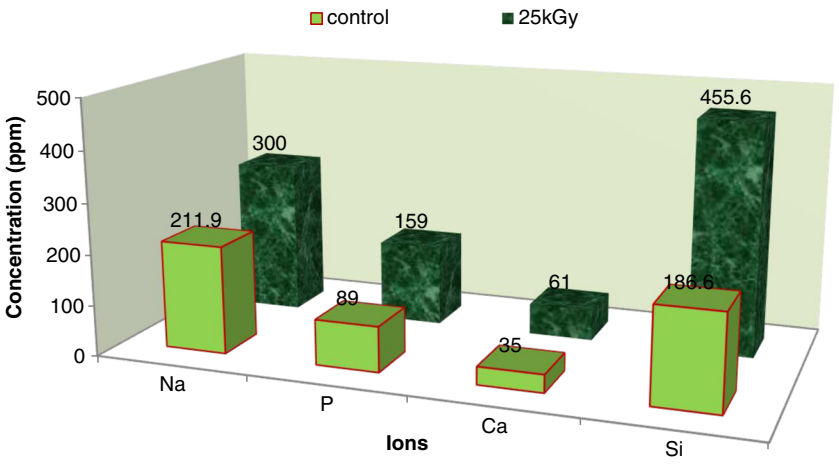

Fig. 9 (ICP-OES) of the ionic concentration in irradiated 45S5 glass at 25 kGy and non irradiated solutions during immersion in the NB

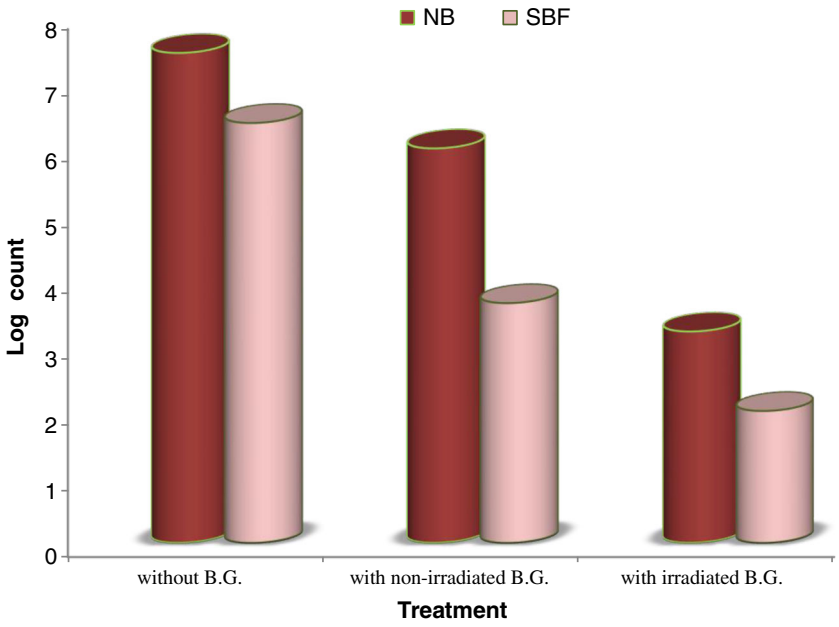

Fig. 10 Anti- biofilm formation activity of irradiated and non-irradiated 45S5 glass against *Ps. aeruginosa*

high dose. It seems that the induced defects are limited to optical spectroscopic investigations.

6.4 Application of Microbiological Studies

Many techniques have been used to sterilize biomaterials including gamma irradiation, autoclaving and gas sterilization. Ionizing radiation kills all types of microorganisms and usually has enough kinetic energy for useful penetration into solids and liquids. 25 kGy is the most common dose for sterilization of biomedical properties [32]. The tested 45S5 glass was submitted to 25 and 50 kGy of gamma irradiation and its activity against the applied strains, compared to non-irradiated 45S5 glass, was evaluated. The data revealed that there was a notable antimicrobial activity of the irradiated 45S5 glass, more than the non-irradiated one.

The exact mechanisms of the antibacterial action of the glass were unknown. High pH and osmotic effects caused by the non-physiological concentration of ions dissolved from the glass have been suggested [33, 34]. Meanwhile, Munukka et al. [35] reported that the better activity of smaller granules could be related to an increase in surface area. The increased surface area would increased the contact of the bioactive glass with the aqueous environment, and so increaseed the release of ions from the glass,which resulted in raising of the local pH and osmotic pressure (The particle size of 45S5 glass particles under study was <45 μm). Palza et al. [36] reported that the ions released from 45S5 glass might penetrate into the bacterial cell,which disrupted the process of DNA replication, or associated with the accumulation of ions in the cell membranes of bacteria, and thus caused a change in their permeability.

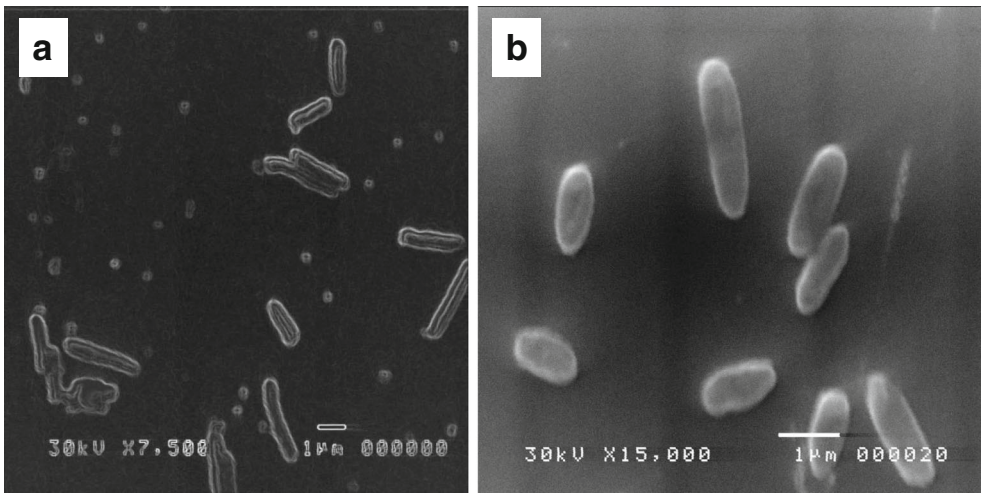

Fig. 11 Scanning electron microphotography of *Ps. aeruginosa*: (**a**) normal cells and (**b**) cells after treatment with irradiated 45S5 glass

In general, Gram negative bacteria were more resistant to 45S5 glass (non-irradiated and irradiated) than Gram positive ones; this was associated with the nature of its outer membrane which brought about intrinsic resistance to a variety of antimicrobial compounds.

Stoor et al. [34] reported that the antimicrobial activity of 45S5 glass was pH dependent. So, the effect of irradiated 45S5 glass in two different pH values of the medium was applied against the tested strains, the release of alkaline ions from the irradiated 45S5 glass caused a rapid increase in the pH and osmolarity of the surrounding environment, making it unsuitable for bacterial growth. The pH change depends on bioglass composition, surface area and irradiation dose and was essential for the antibacterial properties of bioglasses [2, 37].

SBF is a solution that simulates human blood plasma with ion compositions similar to human blood, but without any proteins, hormones, glucose, or vitamins [38]. During immersion in SBF, different processes occur simultaneously which result in structural and chemical changes to the surface of the material. These processes are leaching, degradation, and precipitation [39]. When 45S5 glass is irradiated, it is ionized and exhibits many defects due to electron excitation; when immersed in SBF its dissolution rate increased. As the ions release increased, consequently, the antimicrobial activity increased, as previously reported [27]. Ion release concentrations from 45S5 glass before and after exposure to gamma irradiation were estimated. It was found that gamma irradiation enhance the release of the tested ions. Earlier studies performed by Stoor et al. [40] on S53P4 bioactive glass showed that the amount of silica was tripled during 10–60 min incubation in contact with saline. Regarding the amount of Ca, P and Na, no change was noticed during the incubation time. Also, Zhang et al. [41] concluded that the concentration of Si showed a fast increase from 0 to 160 ppm in contrast to that of P which showed a decrease to less than 5 ppm. So, using irradiated bioactive glass might increase particle solubility and enhance the release of such ions as calcium, phosphorus and silica, thereby increasing the pH level [42].

The formation of biofilms is an important strategy used by microbes for survival. Biofilm producers are extremely resistant to antibiotics as standard antimicrobial treatments typically fail to eradicate biofilms, which can result in chronic infections. So the antibiofilm activity of 45S5 glass (non-irradiated and irradiated at 25 kGy) was investigated. The results obtained showed that the irradiated bioglass suppressed *P. aeruginosa* biofilm formation on AISI 316L discs more than the non-irradiated one. Coraca-Huber et al. [14] reported that S53P4 bioactive glass could suppress *S. aureus* biofilm formation on titanium discs *in vitro*. The suppression rate of biofilm cells by such a glass of particle size <45 µm was significantly higher than by the same bioactive glass 0.5–0.8 µm. The possibility bacterial colonization is a remarkable problem in the use of medical devices. Modifications of the surfaces of devices, for example, by coating them with a suitable bioactive glass, or use of it as a bone substitute in joint replacement revisions may prevent bacterial adhesion and thus prevent the tissues around them from becoming infected [35].

In order to achieve an understanding of the effect of irradiated 45S5 glass on bacterial cells. The morphology of normal *P. aeruginosa* cells and treated ones was observed. Examination of SEM micrographas showed that the morphology of the treated bacteria changed after 3 h of incubation; these results were in concurrence with that obtained by Drago et al. [2], who found that S53p4 bioactive glass formulations displayed a high antimicrobial activity and observed cells were damaged when treated with S53P4 bioactive glass using SEM. Also, the same authors reported a mechanism of action that correlates well with their results of antibacterial activity, showing cell death after 48 h of treatment. The mechanism of action of bioglass is probably double, due to osmolarity and pH variations. Normally, the concentration of solutes within the bacterial cytoplasm is higher than the environment, resulting in a positive pressure on the cell membrane. An increase in external solute concentration (hyperosmotic shock) causes fast water efflux and a pressure drop across the cell membrane, resulting in altered cell size, cell shape and membrane stress levels [43]. All these findings indicate that irradiated 45S5 glass possesses antibacterial activity and it causes lysis and death of bacteria by degrading bacterial cell walls.

7 Conclusion

This study concluded that gamma irradiation along with an alkaline pH and SBF media improves the antimicrobial activity of 45S5 glass. This is perhaps due to exposure to gamma irradiation increasing the rate of ions release that is confirmed by decrease in energy gap. The dose of 25 kGy can be recommended as an effective dose to enhance the antimicrobial activity of such a glass and eradicate the biofilm growth of MDR *P. aeruginosa* in a medium simulating human blood plasma (SBF). This allows application of irradiated 45S5 glass as a bone substitute in bone replacement.

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