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RESEARCH PAPER

The Effect of Gamma Rays on Gelatin / Chitosan Wound Dressing Physico-Chemical and Biological Properties

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Abstract: *In this research, Gelatine (GEL)/ Chitosan (CH) wound dressing was prepared and irradiated with gamma rays from ^{60}Co source for wound healing applications. GEL-CH composite characterization and functional properties were determined. The structural changes occurring after γ-irradiation at doses from 5 to 25 kGy were studied by using physico-chemical techniques such as Electron Paramagnetic Resonance (EPR), Fourier Transform Infrared spectroscopy (FTIR), X-ray diffraction (XRD) and Electrochemical Impedance Spectroscopy (EIS) studies. The antioxidant capacity was studied using DPPH (1, 1-diphenyl-2-picrylhydrazyl free radical) scavenging and the antibacterial activities of Staphylococcus aureus and Escherichia coli were measured using liquid medium. Results revealed that EPR spectroscopy of un-irradiated GEL-CH showed 2 paramagnetic centers associated with g= 2.077 and g= 2.079. After irradiation, no active centre was appeared. A dose-dependent decrease in the central signal intensity was detected, and the EPR signal intensity almost disappeared at 20 kGy. Gamma rays caused a slight increase in ionic conductivity. FTIR suggested a slight crosslinking phenomenon at 20 kGy. The XRD analysis did not show peaks indicating crystallinity in the 2θ range of 15– 30°. Moreover, γ-irradiation elevated the Scavenging DPPH radical activity (0.75 ± 0.07%). Gamma rays did not affect the antibacterial activity of GEL-CH wound dressing against pathogenic bacteria. The results showed that the required γ-radiation for sterilization ranged from 5 to 25 kGy. The radiation improved the physico-chemical and biological properties while maintaining the native structural integrity of the GEL/ COL wound dressing.*

Keywords: *Gelatin, Chitosan, Wound Dressing, γ-irradiation, Electron Paramagnetic Resonance, X-Ray Diffraction.*

1. INTRODUCTION

Gelatin (GEL) is a very attractive biomaterial wound healing applications because they can retain cells and carry bioactive molecules such as growth factors. GEL has been classified as a potent scaffold which interacts perfectly with cells, due to its similar structure to the extracellular matrix. So the GEL scaffold is able to enhance the vascularization process within the newly engineered tissues at the healing site [1]. GEL has been proven to not induce cytotoxic effects, particularly in human cells [2]. Moreover, GEL aids in cell delivery and distribution by creating a microenvironment for the uniform spreading and proliferation of cells [3]. However, in spite of its significant role in wound re-epithelization and healing, using GEL tissue engineering applications is restricted due to its fast biodegradation, poor mechanical properties. Also, native GEL does not show any antibacterial effect [4]. However, to give it such an effect, GEL can be easily integrated with some polymers such as chitosan (CH). This latter molecule is a natural cationic polysaccharide consisting an important tool in research fields due to its significant biological properties such as biocompatibility, biodegradability [5], low toxicity, antioxidant, anti-cancer and antimicrobial properties [6]. The antimicrobial activity of CH is based on the presence of the amino groups of the polymer chain. So, CH is widely regarded as a bioactive substance with reactive functional groups [7]. CH provided ample opportunities for its potential applications imparts to different aspects of wound healing. In fact, CH was applied as a dressing to third degree burns in mice that had been infected with *bioluminescent P* and *aeruginosa and P.*

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mirabilis [8]. Generally, traditional wound dressing products are dry and fail to provide a moist environment to the wound. So that, they have been replaced by modern dressings with more advanced formulations. Polymers of these materials are used alone or in combination depending on the nature and type of wound. Biological dressings are sometimes incorporated with growth factors and antimicrobials to enhance wound healing process.

Herein, the association between GEL and CH can be considered as a sustainable alternative to develop new materials, reducing environmental problems by creating new co-products. One of the major problems with polymers such as GEL and CH is to maintaining the native structural integrity of the material. The question raised was whether the minimum dosage of γ-radiation required for sterilization will damage it. Therefore, it is important to evaluate radiation effects at 5– 25 kGy doses that are used normally in the sterilization of pharmaceutical and medical products [9]. The study incorporated CH into the GEL to take advantage of physico-chemical and biological properties such as antibacterial activities. The research objective is to develop and to characterize the effect of gamma rays on a relatively inexpensive wound dressing that can be used to heal burns injury.

2. MATERIAL AND METHODS

2.1. Pre-treatment of Bovine skin

Skin was soaked in 0.5 M NaOH with ratio of skin/solution at 1:5 (w/v) for 3 days to remove non-collagenous proteins. The solution was changed every day. The alkaline-treated skins were washed with tap water until neutral pH. The samples were then soaked in 0.1 M citric acid, a ratio of 1:5 (w/v) for 1 hour. The samples were again washed with tap water until neutral pH [10].

2.2. Extraction of gelatin

The swollen skin was mixed with distilled water at 1:5 (w/v) at 60°C for 9 h. The mixtures were then filtered using filter paper to remove insoluble materials. The supernatant was freeze-dried and used to prepare a bioactive wound-healing dressing.

2.3. Chitosan preparation

CH (90% DD, degree of deacetylation) was also purchased from Sigma-Aldrich; A 2.0 wt %. CH was prepared in acid acetic solution.

2.4. Preparation of GEL/CH wound healing dressing

The GEL solutions with concentration of 5.0 wt% were prepared by dissolving 5 g GEL powder in 100 ml distilled water for 30 min and then heated at the 50°C for 30 min under continuous stirring. CH solution (2.0 wt%) was prepared by dissolving 2 g CH powder in 100 ml 1% (v/v) acetic acid, stirred overnight at room temperature. All the polymer solutions were filtered through 0.55μm pore-size PTFE membrane filters to remove impurities. It should be noted that no plasticizer was added in the solutions. All mixtures were warmed and stirred at the 50°C for 30 min to obtain a homogenous solution. Finally, the mixture was prepared through repetitive freeze-thawing for four cycles [11].

2.5. Physico-chemical characterization

2.5.1. Gamma irradiation

The irradiations of the GEL-CH composite was performed at the Tunisian Cobalt-60 gamma irradiation facility with energies of 1.173 and 1.332 MeV at a dose rate of 36 Gy/min. The dose rate was determined using Fricke dosimeter chemical standard dosimeter [ISO/ASTM2002c]. The traceability to Aerial, the Secondary Standard Dosimetry Laboratory (SSDL), was established using the Alanine/EPR dosimetry system. Gelatin was placed on a polystyrene phantom to ensure electronic equilibrium and was irradiated at room temperature (293– 298 K) with a dose range from 5 kGy to 25 kGy.

2.5.2. Electron Paramagnetic Resonance Spectroscopy (EPR)

The electron paramagnetic resonance (EPR) spectra of the gelatin samples were recorded at room temperature on a Bruker ER-200D spectrometer operating at the 9.8 GHz X-Band frequencies with modulation amplitude of 0.2 mT , modulation frequency of 100 khz, sweep width of 210 mT and microwave power of 63 mW.

2.5.3. The X-ray diffraction (XRD)

The X-ray diffraction analysis of the GEL-CH composite was conducted using Brucker D8 advance with Cu-Kα radiation of wavelength λ= 1.541 Å in 2θ values in the range of 15– 90° with a step size of 0.02° and counting time of 12

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s per step. The results obtained by X-ray measurement were analyzed with the X'Pert High Score Plus program.

2.5.4. Fourier transform infrared (FTIR)

FTIR spectroscopy is used to study the different functional groups of un-irradiated and irradiated GEL-CH composite. The measurement was recorded at room temperature by Vertex 70 infrared spectrometer from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} at a spectral resolution of 2 cm^{-1} and 32 scans [12].

2.5.5. Determination of Solid polymeric electrolytes

The electrolytes were prepared according to the following formula: 2 g of the composite was dispersed in 15 ml of water and heated under magnetic stirring for a few minutes up to 50°C for complete dissolution. Next, 1.25 g of glycerol as plasticizer, 0.25 g of formaldehyde and acetic acid (36 wt%) were added to this solution with different dose of gamma rays under stirring. This viscous solution was then cooled down to 30°C and poured on Petri plates to form transparent films.

2.6. Biological Analysis

2.6.1. Measurement of Free Radical-Scavenging Activity

The free radical-scavenger activity of the GEL-CH composite was determined by the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, as specified by Koleva et al. [13]; 1 ml of 60-µM DPPH in ethanol was added to 1 ml of different concentrations of the compound. After 30 min of incubation at room temperature, the absorbance was read at 517 nm. The inhibition of nitroblue tetrazolium (NBT) reduction by photochemically generated O_2^- [14] was used to determine the superoxide anion-scavenging activity.

2.6.2. Antimicrobial Activity Test of bioactive wound-healing dressing

Staphylococcus aureus (ATCC25923) and *Escherichia coli* ATCC25922 Gram-positive and Gram negative bacterium respectively, can cause serious infections and has been chosen as a model microorganism in this study [15, 16]. From a young culture on agar, a dense bacterial suspension superior to 10^{6-} Colony-forming unit (CFU) cells/ml) is prepared by dissociating three to five colonies in a 5 ml of sterile saline solution; the density of the suspension was measured using a spectrophotometer (Shimadzu UV mini 1240). The samples of different films were immersed into the saline solution containing the microorganism and stirred at room temperature for 24 h. After that, the number of colonies was counted. All glass wares were sterilized in the autoclave at 120°C for 20 min before experiments.

3. RESULTS

3.1. Electron Paramagnetic Resonance Analysis (EPR)

EPR spectroscopy detects paramagnetic substances such as radicals. Each of un-irradiated and irradiated GEL-CH material were put in a magnetic field and exposed to high-frequency electromagnetic waves and the characteristic of radical show the EPR spectrum. Signal intensity reflected the total absorbed energy of samples under resonance conditions (i.e., the signal intensity was in direct proportion to the number of radicals in samples).The existence of free radicals in polymers is responsible for the appearance of a variety of effects in the polymer chains. EPR has been used to interpret the free radicals generated by the irradiation of polymeric materials with gamma irradiation at doses (5- 25 kGy). Figure. 1 compares EPR spectra of the GEL-CH composite irradiated from 5 up to 25 kGy by ^{60}Co γ-ray with the unirradiated one. It contains 2 peaks which correspond to g= 2.077 and 2.079 respectively. After irradiation, no active centre was appeared. EPR of all samples showed a dose-dependent decrease in the intensity of the central signal until the disappearance at 20 kGy.

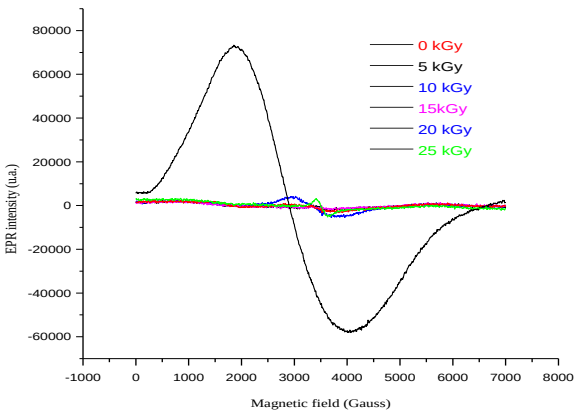

Fig. 1. EPR spectrum of irradiated gelatine/chitosan (GEL/CH) composite with different γ-radiation doses

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5, 10, 15, 20 and 25 kGy in comparison with control samples (0 kGy).

3.2. Fourier transform infrared (FTIR)

For characterizing the chemical changes occurring in the irradiated GEL-CH samples; FT-IR diagrams were achieved at 4000– 400 cm^{-1} wavelength and evaluated for the characteristic peaks (Figure 2). As shown in the spectra, all samples exhibited a broad band around 3425 cm^{-1} and a weak peak at 2920 $cm^{-1,}$ assigned to the groups of -OH and -CH2-, respectively [17]. The bands at 1640 cm^{-1}, 1540 cm^{-1} and 1240 cm^{-1} corresponded to the C=O stretching of amide I, N–H bending of amide II and N–H bending of amide III, respectively [18]. The chemical composition of irradiated GEL-CH samples consists of the same functional groups. However, there was a change in the intensity of the amide II region 1540 cm^{-1} and that of the amide I region 1640 cm^{-1}. Following irradiation, the weak peak at 2920 cm^{-1} was accentuated. The hydrogen bonds inside GEL were substituted by the strong intermolecular interactions between carboxyl and amide groups [19].

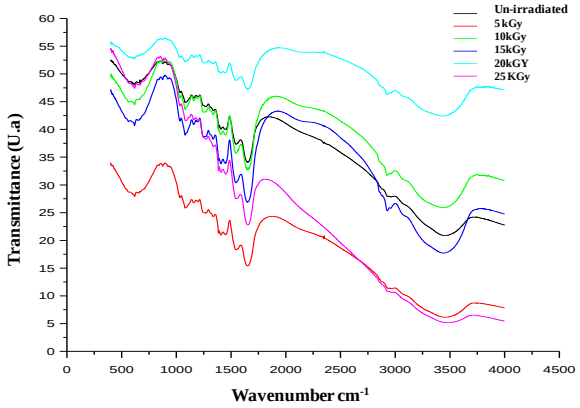

Fig. 2. FTIR spectrum of irradiated gelatine/chitosan (GEL/CH) composite of functional groups with different γ-radiation doses 5, 10, 15, 20 and 25 kGy in comparison with control samples (0 kGy)

3.3. X-ray diffraction (XRD)

The XRD pattern showed different peaks attributed to the composite GEL-CH. In fact XRD pattern showed semi-amorphous morphology with a characteristic broad hump in the range of 15-30 • 2θ. These characteristic peaks are usually assigned to the triple helical crystalline structure in GEL [20]. The XRD reflection revealed a sharp crystallographic peak of CH at 22.0 [21]. After irradiation, the XRD exhibited at different dose level, four sharp, distinct peaks at 2θ= 10.30°, 20.44°, 29°and 38° that represent the distinctive functional peaks of semi-crystalline composite. After irradiation, there was a slight shift in functional peaks of composite. The slight increase in the intensities of functional peaks after irradiation could also be ascribed to the absence of the GEL-CH. crystallinity. The crystallization behavior of the GEL-CH material did not affect or break the originally ordered atomic arrangement with gamma irradiation up to 25 kGy dose.

3.4. Electrochemical Impedance Spectroscopy (EIS)

EIS spectroscopy measurements were used to determine the electrolyte ionic conductivity**.** The ionic conductivity of the GEL-CH electrolytes as a function of gamma ray dose at room temperature was obtained at an acetic acid concentration of 36.4 wt. % as presented in figure. 3. The dependence of ionic conductivity on the ray doses provides information on the specific interaction between the salt and the polymer matrix. It is found that the ionic conductivity increased from of 8.66 × 10^{-4} S/cm at 5 kGy dose to 9.97 × 10^{-4} S/cm at 25 kGy dose. The analysis demonstrated that exposure to ionizing radiation was produced dose-dependent changes.

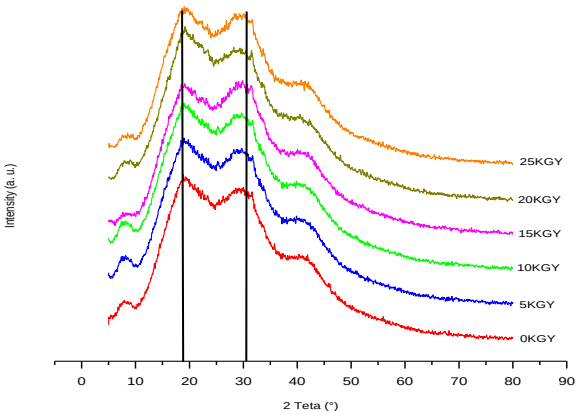

Fig. 3. X-ray diffractograms of irradiated gelatine/chitosan (GEL/CH) composite of with different γ-radiation doses 5, 10, 15, 20 and 25 kGy in comparison with control samples (0 kGy)

3.5. Antioxidant activity assessed by the DPPH radical scavenging test

Gamma rays increased antioxidant capacity of the GEL-CH at doses ranged between 5 and 25 kGy

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(Figure. 4). The irradiation improved the scavenging ability of the composite at different doses compared to corresponding un-irradiated samples. Data showed that the samples treated with 25 kGy had the ability to reduce the stable purple colored radical DPPH into yellow- colored DPPH-H (0.75% ± 0,034) as compared to corresponding control.

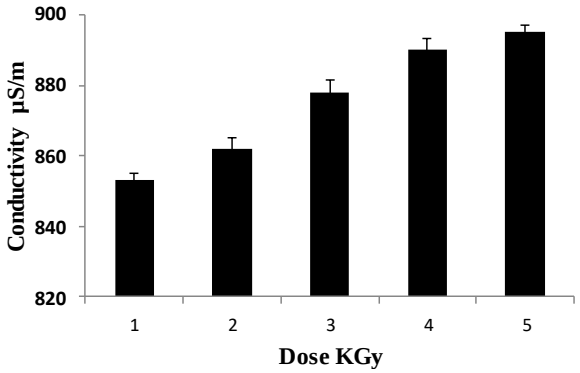

Fig. 4. Impedance plots for gelatine/ chitosan (GEL/CH) composite containing 36.4 wt.% of acetic acid at different γ-radiation doses 5, 10, 15, 20 and 25 kGy in comparison with control samples (0 kGy)

3.6. Antimicrobial activity of

To evaluate the antimicrobial activities of the GEL-CH, we performed tests with a pathogenic bacterium *Staphylococcus aureus and Escherichia coli.* The results of the quantitative antimicrobial tests in a liquid medium are presented in table 1. These results demonstrate that the GEL-CH material showed a considerable antibacterial action against *Staphylococcus aureus* at (5, 10 and 15 kGy doses). However, for *Escherichia coli* the growth inhibition of bacteria was observed at a higher dose (25 kGy). The difference in the results may be due to the differences in cell wall thickness within the group of Gram negative and Gram positive leads to a difference in gamma ray sensitivity [22].

4. DISCUSSION

ESR spectra of irradiated samples from 5 up to 25 kGy dose of radiation demonstrated g-values of the radicals responsible for the spectra between g= 2.077 and g= 2.079. When GEL-CH is irradiated with gamma rays, dissociation of OH groups and degradation occurs through the cleavage of C-O-C bonds producing primary radicals. Some of the primary free radicals undergo a secondary process like dehydration and water elimination leading to the formation of double bonds creation.

For GEL-CH irradiated with up to 25 kGy, negligible amounts of cross-linking are observed. This long polymeric chain has some free groups, such as, hydroxyl, carbonyl groups. groups, etc. Those groups can produce radicals and new chemical groups. The similarity of peak intensity at 895 and 1151 cm^{-1} also characteristic of saccharide structure, suggest that the CH β-glucosidic linkages remain stable after γ-irradiation. On the other hand, the conductivity varies with a wide range of factors, such as cation and anion types, salt concentration, temperature and irradiation [21]. One Study on the GEL and CH interactions showed that two kinds of interactions can give rise between both polymers when they are in contact with water: an electrostatic complex and a hydrogen bonding type complex [23]. The polycationic characteristic of the CH molecule is the responsible for the higher electric conductivity compared to the GEL. A polyanion–polycation complex is present between the two polymers [23]. The increase in the ionic conductivity with increasing doses can be related to the increase in the number of mobile charge carriers [24].

In fact, the gamma rays induced by radiation scission of CH molecules and hydrolysis of the samples to produce more ions, OH^-, and H^+ that are trapped in localized sites in the GEL-CH matrix. An increase in gamma dose might result in scission of biopolymer chains and lead to a slightly increase in conductivity that may be explained that cross-linking of biopolymer chains is not completely enough. On the other hand, the antioxidant activities were evaluated under different treatment of the acute gamma irradiation (up to 25 kGy). The data showed increases of the scavenging ability of the DPPH radical irradiated samples.

The dose of 20 and 25 kGy had the highest activities as compared to that of the un-irradiated sample. According to one study, [25] the use of CH as antioxidant additive had been reported in numerous researches and had demonstrated a great capacity for interacting with free radicals through ionic interactions with its amino group. On the other hand, as known [25]. GEL did not exhibit antibacterial activity against the test microorganisms. In the present study, the

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incorporation of CH into GEL showed significant antibacterial activity essentially against *Staphylococcus aureus* and *Escherichia coli*.

Table 1. Effects of active gelatine/chitosan (GEL/ CH) based on antibacterial activity: Viable cell counts after different doses of irradiation.

	No . of colonies (CFU/ml)					
	0kGy	5 kGy	10 kGy	15 kGy	20 kGy	25 kGy
Staphylococcus aureus	$>10^6$	$>10^5$	$>10^5$	$>10^5$	$>10^6$	$>10^6$
Escherichia coli	$>10^6$	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$

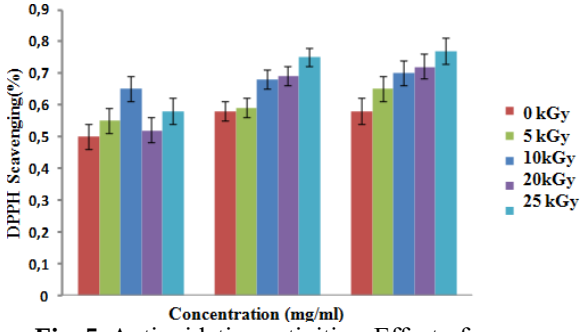

Fig. 5. Antioxidative activities; Effect of gamma irradiation doses on DPPH scavenging activity irradiated gelatine/ chitosan (GEL/CH) composite of with different γ-radiation doses 5, 10, 15, 20 and 25 kGy in comparison with control samples (0 kGy)

The antibacterial mechanism could be due to the interaction with lipophilic components of the bacterial membrane with a changes in the permeability of H^+ and K^+, and finally damage the essential functions and cause cell death [26]. The incorporation of CH conferred some antimicrobial activity that prevents bacterial strains. One of the reasons for the antimicrobial character of CH is its positively charged amino group which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms [27, 28]. In addition, some proposed mechanism is the binding of CH with microbial DNA, which leads to the protein synthesis via the penetration of CH into the nuclei of the microorganisms [29, 30]. CH molecules are assumed to be able to pass through the bacterial cell wall, and reach the plasma membrane. The research confirmed the presence of CH oligomers inside *E. coli* under various circumstances. In the present study, there was no significant change found in either type of antibacterial activity resulting from gamma irradiation (form of sterilization) of the GEL-CH, even when the radiation reached 25 kGy.

5. CONCLUSIONS

The study reported the preparation and evaluation of GEL-CH material for wound healing activity. The EPR signals recorded in the polymeric chain were due to some free chemical groups, such as hydroxyl groups in the biopolymer chains. The ionizing radiation enhanced the electrical conductivity of the composite sample. After gamma rays irradiation, FTIR indicated a slight crosslinking phenomenon. The XRD analysis showed no change in crystallinity. Moreover, an improvement in the antioxidant performance without altering antimicrobial properties of the GEL-CH composite after γ-irradiation was noticed. The prepared GEL-CH composite sample could provide a favorable environment to promote healing process while protecting the area from infection. Therefore, the innovative results provides a useful reference to the application and usefulness of gamma irradiation for pharmaceutical sterilization.

6. CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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