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Influence of some factors affecting antibacterial activity of PVA/Chitosan based hydrogels synthesized by gamma irradiation

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Abstract Poly (vinyl alcohol) hydrogels containing different concentrations of chitosan with molecular weight of 471 and 101 kDa were crosslinked by gamma irradiation at a dose of 25 kGy. The swelling behavior, gel content and morphological structure of the blend were investigated. The antibacterial effect, as a function of chitosan content and molecular weight in the hydrogel, was investigated against *Escherichia coli* and *Bacillus subtilis.* With increasing chitosan content the equilibrium degree of swelling of the blend increased and the gel fraction decreased. Results of antibacterial activity of chitosan revealed that chitosan was more effective in inhibiting growth of gram positive bacteria than that of gram negative ones. It was observed that, the chitosan content as well as its molecular weight has a direct influence on bacteria growth inhibition. The higher the chitosan content in the blend and the higher its initial molecular weight, the larger was the inhibition zone diameter. The bacteria growth inhibition was attributed to the diffusion of entrapped chitosan from the hydrogel blend to the culture medium.

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1 Introduction

In recent years, much interest has been shown in the development of wound dressings based on polymer blends. In order to fit the exigencies of the purpose for which they are intended, the polymer dressings must be transparent, permeable to water and oxygen, but impermeable to bacteria [1], moreover it should provide a moist and healing environment for the wound and be biocompatible [2]. A large number of hydrogels developed exhibit such properties and have been considered to be advantageous in their application as a wound dressing material [3–5].

Hydrogels are two or multicomponent polymeric networks that can swell without dissolving in water [6, 7]. They are mainly based on water soluble polymers, such as poly (vinyl alcohol) (PVA), poly (vinyl pyrrolidone) (PVP), poly (acrylic acid) (PAA) and poly (ethylene oxide) (PEO). During the last decade very promising materials for wound dressing have been synthesized, with PVP [8–10], PVA [11], PEO [12] and polysaccharide like chitosan, alginate, collagen, and cellulose [13, 14].

Among the methods applied to prepare hydrogels, radiation technique is a very convenient tool, as a simple and reliable process that did not require any additives to crosslink the polymer or to initiate polymerization reaction. Moreover by this method the processed materials are also simultaneously sterilized [9, 15].

The continuous search for hydrogels with improved specific properties was oriented toward the development of composites of synthetic and natural materials. Polysaccharides are interesting materials that can be explored in combination with synthetic polymers for developing hydrogel systems [12].

PVA is a synthetic polymer used in a large range of medical, commercial, industrial and food applications, manufacture of paper products, surgical threads, wound care and food-contact applications [16]. It was recently used as a coating for dietary supplements and pharmaceutical capsules [17]. Cross-linked PVA was also used for controlled release of oral drugs [18].

Chitosan is a polysaccharide that is derived from chitin, a major component of the crustacean exoskeletons. It is a non toxic and biocompatible cationic polysaccharide produced by partial deacetylation of chitin. Its properties provide high potential for many applications [19]. Chitosan has been widely used in diverse fields, such as in biomedical applications [1, 20], drug delivery, in agriculture [21] metal ion sorption [22]. The most important characteristic of chitosan is the deacetylation degree (DD) which influences its physical and chemical behaviors [23].

Chitosan extracted from squid pen chitin is inherently purer than crustacean chitosans; it does not contain large amounts of calcium carbonate. The purity of squid pen chitosan makes it particularly suitable for medical and cosmetic application [24, 25].

Recently, the blends between PVA and chitosan were widely investigated due to their potential use in medical and environmental fields [26–29]. It is well known that the chitosan and modified chitosan derivatives have antibacterial and antifungal activities [30–34] and that this property depends of the molecular weight of chitosan [35]. Application of radiation for the formation of hydrogels through cross-linking for medical use offers a unique possibility to combine the formation and sterilization of the product in a single technological step [9, 36, 37].

The aim of this study was to synthesize wound dressing, using gamma irradiation a PVA based hydrogels containing different concentration of chitosan of two different molecular weights and to investigate their antibacterial activities and the characteristic of the prepared PVA/Chitosan based hydrogels was studied in detail.

2 Materials and methods

2.1 Materials

Chitosan Cs_1 with MW = 471 kDa and DD of 70%, and chitosan Cs_2 with MW = 101 kDa and DD of 90%, were produced locally from squid pens chitin (*Loligo* sp.). PVA purchased from Fluka (MW = 205 kDa), with a degree of polymerization of 4200 and the degree of hydrolysis of 86%, polyethylene glycol (PEG 400) (Fluka) were used without any purification.

2.2 Preparation of the hydrogel

PVA/chitosan blended hydrogels were prepared by dissolving chitosan powder at concentrations ranging from 0.25 to 1% in an aqueous solution of 0.25% acetic acid. PVA powder was added to the chitosan solution to a total polymer concentration of 5%, PEG, as plasticizing agent, was added at 1.5% to the mixture. Whole solution was poured into polyethylene terephtalate (PET) molds of rectangular shape (10 × 7 cm) and 3 mm deep, which were then packed in polyethylene sachets hermetically heat-sealed. The samples were then irradiated with gamma rays to a total dose of 25 kGy with a dose rate of 19.10 Gy/min.

2.3 Swelling measurements

The swelling kinetics was carried out through measurements of water uptake of the hydrogel as a function of time. Hydrogels samples were placed in deionized water at room temperature. The mass of the swollen gel was measured at different intervals of time until equilibrium swelling was reached. The degree of swelling (S%) was determined according to the following relationship:

$$S(\%) = [(W_d - W_s)/W_s] \times 100$$

W_s and W_d represent the weight of hydrogel at equilibrium after and prior to the immersion.

2.4 Gel fraction

Hydrogels were first dried at room temperature for 72 h then in vacuum oven at 60°C until a constant weight (W_{ip}) was reached. The sol fraction was extracted by autoclaving the gels in distilled water at 120°C and 1 bar pressure for 2 h. The extracted hydrogels were dried at 60°C for 48 h to a constant weight (W_{dg}). The gel fraction, Gel (%) was determined as the ratio of the dry gel weight before (W_{ip}) and after (W_{dg}) autoclaving.

$$\text{Gel}(\%) = \left(W_{dg}/W_{ip}\right) \times 100$$

2.5 Scanning electron microscopy (SEM)

The morphological structure of the hydrogels was examined under a Philips XL 30 ESEM SEM. Prior to observation, the samples were dried at room temperature and then coated with thin layer of gold (20 Å).

2.6 Chitosan release

In order to investigate the behavior of chitosan molecules at the interface between the hydrogel and the physiological environment, rectangular pieces (3 cm^2) of PVA/chitosan

hydrogel were put on nutrient agar gel at 37°C for 24 h. Then the hydrogel was withdrawn and the nutrient agar medium was colored by 0.2% Congo Red dye. After few hours, the nutrient agar medium was washed several times with distilled water, and the zone remaining colored was observed.

2.7 Antimicrobial tests

Antibacterial activity of the blend hydrogel PVA/chitosan against *Escherichia coli* (Gram negative) ATCC 10536 and *Bacillus subtilis* (Gram positive) ATCC 6633, were evaluated by using the diffusion method. The microorganisms were obtained from Pasteur Institute of Algeria.

2.8 The preparation of the microbial suspension

The bacteria *E. coli* and *B. subtilis* were inoculated into 20 ml peptone liquid culture medium. Having being inoculated in air bath shaker at 30°C for 24 h, the strain entered the exponential period of the growth and the culture broths were diluted. The concentration of *E. coli* and *B. subtilis* were 10^7 ufc/ml.

2.9 Condition of incubation

Small piece of sterile discs of hydrogels PVA/chitosan ($\sim$0.80 mm^3) were deposited on agar medium (pH = 5.5) which was previously inoculated with a suspension of bacteria. The chitosan will diffuse in the area surrounding each piece of hydrogel and a disc of bacteria lyses will become visible. All samples were incubated at 30°C for 24 h, then the plates were taken out and the inhibition area was determined.

2.10 Statistical analysis

All experiments were replicated at least three times. The results obtained were treated statistically by the calculation of the central tendency and dispersion parameters, and application the Student test for the comparison between the various arithmetic means.

3 Results and discussion

3.1 Swelling measurements

On Figs. 1 and 2 were represented the swelling kinetics of PVA/Cs_1 and PVA/Cs_2 respectively. It can be observed from the curves that, the swelling ratio increases rapidly during the first 10 h, then levels off to reach the equilibrium state after 96 h. The swelling rate of the hydrogel

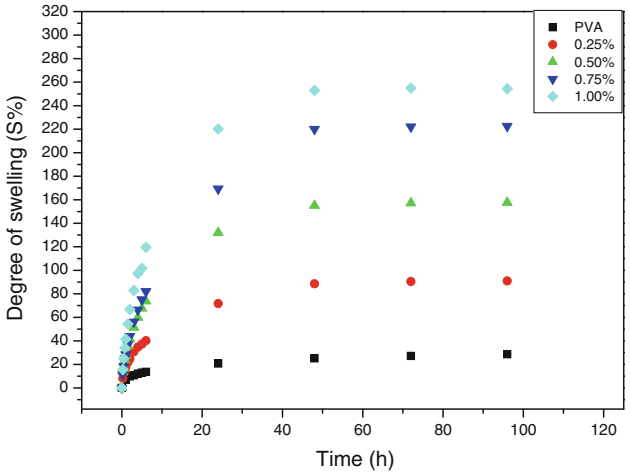

Fig. 1 Variation of water uptake of PVA/Cs_1 vs. time

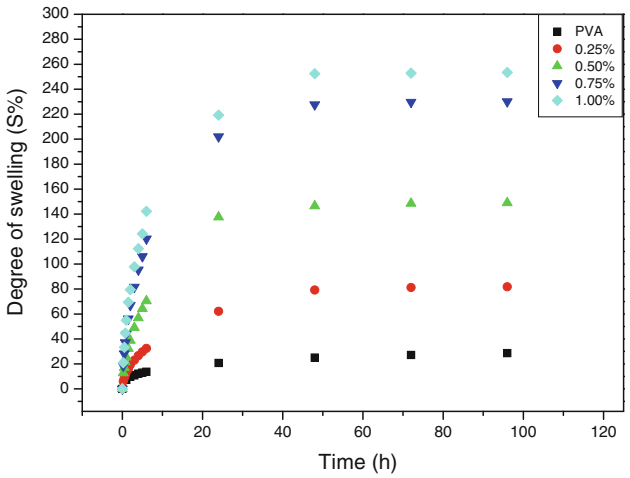

Fig. 2 Variation of water uptake of PVA/Cs_2 vs. time

increases with increasing of chitosan content. The Student test showed that there is a significant difference in the degree of swelling versus chitosan content; the higher the chitosan concentration, the higher was the equilibrium swelling ratio.

Irradiation of PVA in aqueous solution leads to the formation of insoluble polymer network as a result of crosslinking that takes place by recombination of radicals formed on polymer chains by irradiation. PVA hydrogel swells by absorption of water, which is kept in the free volumes of cross-linked polymer. Water uptake is the highest when the network is connected by relatively low number of intermolecular bonds and it decreases with cross-linking density increasing [8].

The presence of chitosan in the polymer solution reduces the probability of radicals recombination, thus the crosslinking density of the gel becomes lower. As a result, more free volumes are available in the polymer network, and consequently more water can be absorbed. So the

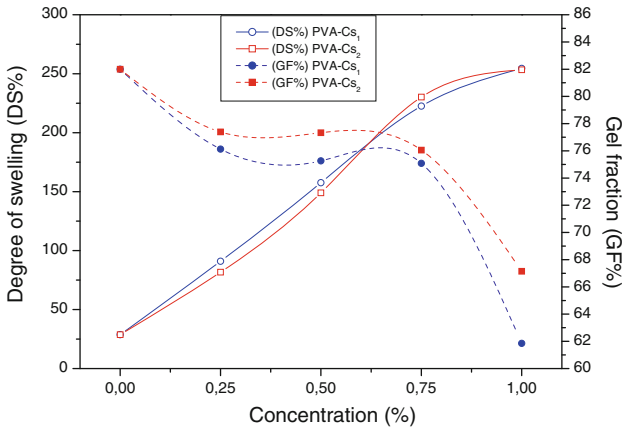

Fig. 3 Gel fraction and degree of swelling after 96 h of PVA/Cs_1 and PVA/Cs_2 vs. chitosan content

higher the chitosan content in the hydrogel, the lower is the crosslinking density, and the higher is the swelling ratio. Chitosan content contributes to the swelling of hydrogels; this could be due to ionic charge of chitosan allowing more ingress of water into the network due to osmotic pressure.

Although a slight difference was observed in swelling ratio of the two blends (Fig. 3), the molecular weight of the chitosan did not seem to have a significant influence on the swelling ratio of the gel.

3.2 Gel fraction

Exposure of certain polymer solutions to ionizing radiation causes gelation because of cross-linking [38]. The mechanism of crosslinking involves the cleavage of C–H bonds in neighboring polymer chains, subsequently; the polymeric radicals recombine to form intermolecular bonds [39]. Cross-linking mechanism of PVA solutions by gamma irradiation has been widely investigated [40–42]. Knowing that, PVA is a polymer of crosslinking type and that chitosan, as a polysaccharide, is more likely to degrade when exposed to radiation [11], when PVA/chitosan blended mixture is exposed to gamma irradiation, cross-linking of PVA and degradation of chitosan to shorter chains take place simultaneously. At the same time occurs to the formation of three-dimensional network of hydrogel [34].

It is well known that in aqueous solution, the indirect effect of radiation is the main interaction mode, i.e. the primary reactions occur with water, producing powerful oxidizing species, such as hydroxyl radicals $^{\cdot}OH$, that can attack the β (1–4) glycosidic bonds of chitosan [43]. Hence, the radiation processing of chitosan in presence of water would reduce significantly its molecular weight. It was shown in the previous study [44] that the irradiation at 25 kGy of chitosan in aqueous solution (acetic acid -sodium acetate) reduced the molecular weight from 471 to 88.3 kDa and from 101 to 17.4 kDa for Cs_1 and Cs_2 respectively.

Varshney [11] reported that, when an aqueous solution of PVA (7–9%) containing polysaccharides (1–2%) is exposed to radiation, $^{\cdot}OH$, $^{\cdot}H$ radicals and hydrated electrons are produced, as major part of the energy is absorbed by the solvent. $^{\cdot}OH$ radicals are mostly responsible for crosslinking of PVA and degradation of polysaccharides, and the rates of $^{\cdot}OH$ radical reaction with PVA and polysaccharides are similar. Therefore, besides crosslinking of PVA, a fraction of radicals would also degrade the polysaccharides in proportion to their concentration in aqueous PVA solution. In our case the chitosan content was less than 20% of the polymers present in the mixture, the yield of radicals generated on chitosan would be less than 20% of that produced in a pure chitosan solution with a same concentration. Consequently the molecular weight of chitosan in the hydrogel was reduced after irradiation.

The gel fraction is the insoluble part of the polymer caused par crosslinking. The variation of gel fraction and degree of swelling of the hydrogels as a function of the chitosan content is shown in Fig. 3. It was observed, that the gel fraction decreases slightly with increasing chitosan content in the hydrogel blends and levels off above the concentration of 0.75%. At higher concentration of chitosan (1%) the gel fraction decreases drastically. High concentration of chitosan hinders the recombination of radicals. The higher the chitosan concentration in the hydrogel, the lower was the gel fraction. It was reported by Varshney [11] that the presence of polysaccharides (carrageenan and agar) in aqueous PVA solutions affects the properties of the gel formed on irradiation.

On the other hand, it can be observed that the gel fraction was higher in the blend containing low molecular weight chitosan (PVA/chitosan Cs_2) than in that with higher one (PVA/chitosan Cs_1). High molecular weight of chitosan induces high viscosity of the blend solution; this reduces the mobility of the PVA radicals formed by irradiation and prevents the recombination reactions between them, as a consequence, the crosslinking yield was reduced. Thus both parameters, concentration and molecular weight of chitosan, affect the gel fraction of the PVA/chitosan based hydrogel.

In addition to the chitosan, the hydrogels synthesized contain, as a plasticizer, PEG which also hinders crosslinking of PVA. This phenomenon was observed for relatively high concentrations [45]. By the control of PEG content, one can control the gel fraction of the hydrogel at a given irradiation dose [46]. In our hydrogels the PEG was added at 1.5% to the mixture, this amount was optimized before in order to fit good requirements, such as high gel fraction, good elasticity and good mechanical properties.

At this concentration PEG did not affect considerably the gel fraction which is about 82% [47].

3.3 SEM

SEM informs us, about the porosity of tri-dimensional network of the hydrogel. The surface texture of PVA and the PVA/chitosan blend were shown in Fig. 4. PVA micrograph showed a uniform and homogenous surface (Fig. 4a), in the blend hydrogels, we can observe a heterogeneous texture with slightly increased porosity, induced by the presence of chitosan in the blend (Fig. 4b, c). In fact, due to the poor hydrophilicity of chitosan, its miscibility with PVA in aqueous solution was weak and consequently, the formation of a homogenous mixture was hindered.

Comparing the Fig. 4b and c, one can conclude that the heterogeneity seems to be enhanced by the high molecular weight of chitosan (Fig. 4c).

On one hand, this difference in the porosity plays a prominent role in swelling behavior of the hydrogel, and on the other hand it can play also an important role in chitosan release from hydrogel network, thus in antimicrobial activity of chitosan.

3.4 Chitosan release

After several washings of the nutrient agar medium, two intensities of coloration were observed; an intensive red coloration situated on the zone which was in contact with the hydrogel, and more clear red color around it (Fig. 5). Knowing that the nutrient agar medium did not adsorb the dye, the permanent coloration is due to the presence of chitosan molecules. In fact, the cationized amino groups ($-NH_3^+$) of chitosan adsorbed Congo red anions ($R-SO_3^-$) by electrostatic attraction. This may suggest that the chitosan, entrapped in the network of the hydrogel, released to the nutrient agar medium. It was not grafted to the PVA.

3.5 Antimicrobial activity

Microbiological test showed that the hydrogels containing different concentrations of chitosan exhibit antimicrobial activities against *E. coli* and *B. subtilis*. Both hydrogels, PVA/Cs_1 and PVA/Cs_2, showed more effective inhibition on *B. subtilis* than on *E. coli*. The effect may be attributed to their cell wall composition. *B. subtilis* is a Gram positive bacterium, its cell wall is composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allow chitosan to come into the cell easily and disturb its metabolism, whereas *E. coli* is a Gram negative bacterium, the cell wall of which is made up of a thin membrane of peptidoglycan and an outer membrane constituted of lipopolysaccharides and phospholipids, that constitute a barrier against chitosan [48].

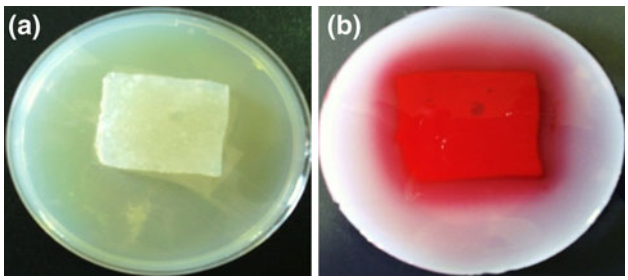

Fig. 5 Chitosan release from blend into the culture medium: **a** before coloration and **b** after coloration

3.6 Effect of chitosan DD

It can be observed also, that the hydrogel containing Cs_1 was more effective in inhibiting both *E. coli* and *B. subtilis* than that containing Cs_2, independently of chitosan concentration (Fig. 6). The Student test showed that, this difference is significant.

Knowing that the DD of Cs_1 (70%) was lower than that of Cs_2 (90%), it was expected that the antibacterial activity of Cs_2 will be higher than that of Cs_1, since at high DD, higher were the free amino groups present in chitosan, and higher should be the antibacterial effect. But the results obtained in the present study are contrary to those expected. This would mean that for these values of DD (70–90%), the influence of molecular weight on antibacterial activity was more dominant than the deacetylation degree.

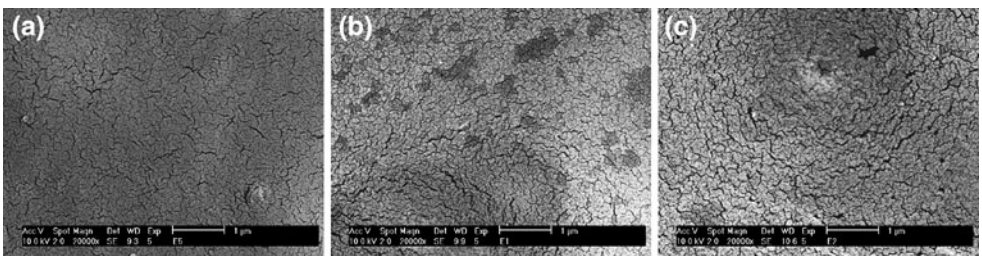

Fig. 4 SEM photomicrographs: **a** PVA, **b** hydrogels PVA/Cs_2 and **c** hydrogels PVA/Cs_1

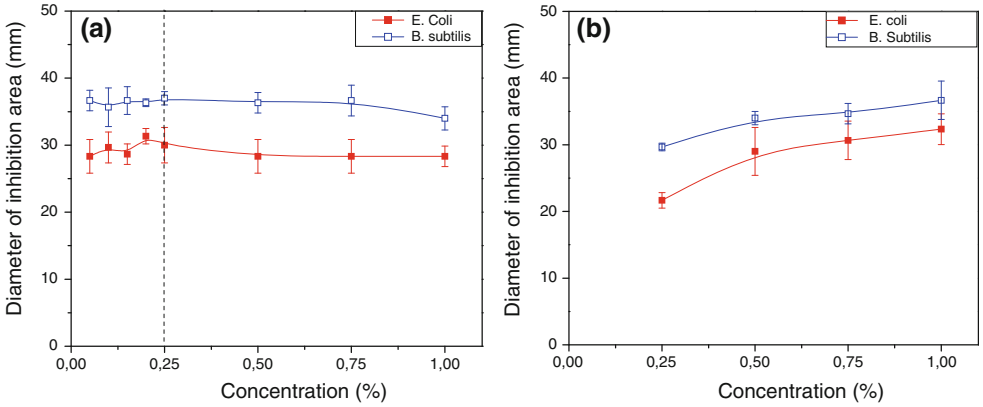

Fig. 6 Diameter of inhibition zone vs. concentration of chitosan: **a** PVA/Cs_1 and **b** PVA/Cs_2

In this study, where the chitosan was trapped in the polymer matrix, its antibacterial effect did not appear to be governed by its degree of deacetylation. Other factors could come into play, including the amount of chitosan having diffused in the culture medium, and therefore its concentration in this environment and certainly its molecular weight.

In our case of study, the antibacterial activity was governed by the diffusion of chitosan from the hydrogel to the culture medium. So if we consider that the lower the molecular weight, the easier would be the diffusion, the Cs_2 would diffuse more than Cs_1 to the culture medium, leading to a higher chitosan concentration in the medium. Despite this, the antimicrobial activity was more pronounced among Cs_1. This leads us to say that the influence of molecular weight on the antimicrobial activity is more dominant than the concentration and DD.

3.7 Effect of chitosan molecular weight

The initial molecular weights of the chitosan incorporated in Cs_1 and Cs_2 were respectively 471 and 101 kDa, but irradiation at 25 kGy could reduce them to a lower values. The radiation degradation of chitosan in aqueous solution is not similar to that in the hydrogel, where it is difficult to measure the molecular weight of chitosan. The molecular weight of Cs_1 and Cs_2 could be reduced to values lying between 471 and 88 kDa and 101 and 17 kDa for Cs_1 and Cs_2 respectively after receiving a dose of 25 kGy. This value of MW is within the range of MW reported to be more effective in enhancing antimicrobial activity of chitosan.

It can be observed also, that the hydrogel containing Cs_1 is more effective in inhibiting *E. coli* and *B. subtilis* than that containing Cs_2, independently of the concentration of chitosan. The Student test showed that, this difference is significant. This effect may be attributed to the fact that the molecular weight of Cs_1 is higher than that of Cs_2.

The antimicrobial activity is not systematically higher for low molecular weight chitosan, but it is for a specific range of molecular weights. This was confirmed by the antibacterial tests we conducted in solid and liquid media with chitosan of respectively $MW_1 = 88.3$ and $MW_2 = 17.4$ kDa. The inhibition diameters in the solid medium were 35 and 28.25 mm for MW_1 and MW_2 respectively. More significant results were obtained in the liquid medium, since the counts were 28×10^2 and 294×10^2 cfu/ml for MW_1 and MW_2 respectively, where the initial bioburden was 615×10^6 cfu/ml. These two tests confirmed that the chitosan of 88.3 kDa MW is more effective in bacterial growth inhibition than the 17.4 kDa one.

Qin et al. [49] studied the antibacterial activity of chitosan in the molecular weight range 1.4–400 kDa, and found that the highest antimicrobial effect was obtained for MW 78 and 48 kDa. Liu et al. [35] reported that the antimicrobial activity is more pronounced for low MW in the range of 55–155 kDa. Lam and Diep [50] studied the antifungal activity of chitosan in the range of MW varying from 72.5 to 139 kDa and found that the highest activity corresponded to the MW of 122 kDa.

3.8 Effect of chitosan concentration

The diameter of inhibition zone for *E. coli* and *B. subtilis* in PVA/Cs_1 did not change with increasing concentration of chitosan in the blend (Fig. 6a); the Student test showed that there was no significant difference in diameters of inhibition zone for the different Cs_1 concentrations from 0.05% to 1%. This may suggests that chitosan release is governed by its molecular weight independently of its concentration. It can be observed also that the blend PVA/Chitosan based hydrogels exhibit antibacterial activity even when the chitosan Cs_1 concentration was as low as 0.05%.

Unlike for Cs_1, with Cs_2, the Student test showed that there was a significant difference in diameter of inhibition zones, with the increase of chitosan concentration, the

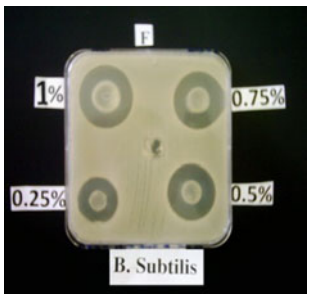

Fig. 7 Inhibition zone of PVA/Cs_2 at different concentrations of Cs_2 on *B. subtilis*

diameter of inhibition zones increased (Fig. 6b). This phenomenon could be attributed to the low molecular weight of chitosan. The amount released increased with the increase of its concentration in the blend, due to its low molecular weight, the chitosan could be released easily to the medium.

On Fig. 7 is shown an example of inhibition zones of PVA/Cs_2 blends. The chitosan concentration in the hydrogel formulation is indicated in percentage. The spot in the middle of the Petri dish corresponds to the PVA hydrogel without chitosan. This figure illustrates the fact that the PVA hydrogel did not inhibit bacteria's growth. The inhibition zone was the result of the presence of chitosan in the hydrogel.

4 Conclusion

The addition of chitosan to the composition of PVA based hydrogels influences some characteristics of the blend, such as its swelling behavior and antibacterial activity. These changes were governed mainly by the concentration and the molecular weight of the chitosan. The release of chitosan from the blend into the culture medium was even more important that its molecular weight was low. But the antibacterial activity of high molecular weight chitosan was higher than that of low molecular one.

Both PVA/Cs_1 and PVA/Cs_2 based hydrogels exhibited an antibacterial activity, which was more effective against gram positive bacteria than against gram negative bacteria. The PVA/Chitosan based hydrogel could be used as wound dressing material.

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