Egypt. J. Rad. Sci. Applic., Vol. **25**, No. 1-2, pp. 77-90 (2012)

Synthesis, Characterization and Anti-bacterial Activity of Ag-NPs

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SYNTHESIS, characterization and comparison the anti bacterial activity of both silver/ polyvinyl alcohol (Ag/ PVA) nanocomposite and nisin, for some pathogenic bacteria was carried out. Applications of the nicin include dental care products, pharmaceutical products such as stomach ulcers and colon infection treatment and potential birth control. Ag/ PVA nanocomposite was prepared by in situ reduction method in which silver nitrate, gamma irradiation and PVA act as precursor, reductant and stabilizer respectively. The synthesized nanocomposites have potential antibacterial activity toward both Gram-positive and Gram-negative bacteria. Further studies have demonstrated the structure and the distribution of Ag nanoparticles caped within PVA polymer chain such as X-ray diffraction (XRD), transmission electron microscopy (TEM) were carried out.

Keywords: Silver nanocomposite, PVA, nisin, antibacterial activity.

Nisin is one of the most nicin which used in practical applications. They can either be added as preservative (nisin is the only nicin so far to be used in this way) or they can be produced *in situ*, *i.e*., in the product in the case of starter cultures or in the gastrointestinal tract in the case of probiotic strains. Some nicin appears to be produced as a product (Bortz *et al.*, 2000), but *in vivo* production is still an open question. *In situ* production of antimicrobial substances in the intestine might be enhanced by increasing the adhesion of the probiotic strains to the intestinal mucosa. However, a potential risk with *in situ* production of nicin in the intestine is that beneficial members of the normal micro flora are affected (Sanders, 1993). Nisin is short peptide antibiotic produced by some strains of *Lactococcus lactis* subsp *lactis*. Nisin is non-toxic to humans and is readily broken down by digestive enzymes when consumed.

Hypersensitivity to nisin has not been recorded so, it has used as a food preservative in other countries since 1954 and has recently gained approval in the United States for use in certain dairy products (Broadbent *et al.*, 1989). Molecular formula $C_{143}H_{230}N_{12}O_{37}S_7$, molecular wt 3354, normally nisin occurs as a dimmer with molecular wt 7000 (Jarvis, 1970).

Nicin has large antimicrobial activity spectrum against Gram-positive bacteria and their spores, but shows little or no activity against Gram negative bacteria, yeasts or moulds. However, Gram negative bacteria can be sensitized to nisin by exposing to chelating agents ethylene di-amine tetra acetic acid (EDTA), sub lethal heat and freezing (Vessoni Penna *et al.*, 2000). Nisin has been accepted as a safe and natural preservative in different areas of food industry and it has also been used as treatment for some health conditions such as stomach and colon ulcers, cosmetic and veterinary products (Von Sataszewski and Jagus, 2008).

Recently, the investigation of the attractive antibacterial activities of silver nanoparticles (Ag-NPs) has reclaimed importance due to an increase of bacterial resistance to antibiotics caused by their overuse. Presently, Ag-NPs displaying antibacterial activity are being synthesized. Antibacterial activity of the silver-containing materials can be used, for example, in medicine to reduce infections as well as to prevent bacterial colonization on prostheses, dental materials, vascular grafts, catheters, human skin, and stainless steel materials (Panacek *et al.*, 2006).

Many methods, such as chemical reduction, and radiolytic method have been employed for the synthesis of Ag-NPs (Mnniz Miranda, 2004). These methods are expected to result in a narrow particle size distribution and particles of uniform shape. Since the metal colloids tend to coagulate, they are usually unstable and difficult to be used. As a result, their antibacterial activities are poor. This problem can be greatly overcome by embedding or encapsulating the metal nanoparticles with polymer materials (Mbhele *et al.*, 2003). Ag-NPs protected by polymers, such as PVA (Chou and Ren, 2000), poly (vinyl pyrrolidone) (PVP) (Khanna *et al.*, 2004), polystyrene (PS) or poly methyl methacrylate (PMMA) (Monti *et al.*, 2004), have been extensively reported. PVA could be considered as a good host material for metal, due to its excellent thermo-stability and chemical resistance (Porel *et al.*, 2005). In addition, owing

to its water solubility, the Ag-Nps can be easily prepared in aqueous medium and the preparation is virtually non-toxic.

In this work, gamma-radiolytic synthesis of Ag nanoparticles capped with PVA matrix and the antibacterial activity of Ag/ PVA nanocomposites as compared with the nisin was investigated.

Experiment

Preparation of Ag/ PVA nanocomposites

PVA with a molecular wt of 32000 g/mol was used as a basic polymeric material in this work. PVA solution was prepared by dissolving 6 g PVA in 90 ml of distilled water. The solution was warmed up to 85^{o}C and thoroughly stirred by using a magnetic stirrer for 4 h until the polymer become completely soluble.

For the preparation of Ag/PVA nanocomposites film, 10 ml $AgNO_3$ solution (0.1M) was added to the above PVA solution (6 wt % PVA content). Then the solution stains to cool at room temperature. Then the solution was castled in a Petri dish, thereafter the film was irradiated using "Gamma cell 220 Excel ^{60}Co irradiation facility" manufactured by the Atomic Energy Authority of India. The absorbed irradiation dose rate of the γ-cell was 2.857 kGy/ h. A set of test polymeric samples has received the following irradiation doses (25 and 100 kGy). Finally the film was dissolved in deionised water for the antibacterial activity investigation.

Characterization techniques of Ag/ PVA nanocomposite

Ultra Violet (UV) double beam Unicam, ultraviolet/ visible (UV/ VIS) spectrometer made in England was used for scanning the absorption spectra in the range from 190 nm to 800 nm wavelengths and measuring the optical density at λ_{max} for the investigated samples. TEM of the type JEOL JSM-100 CX, Shimadzu Co., Japan was used to Study the shape and to determine the particle size distribution of the nanoparticles. XRD patterns of the samples were performed at room temperature by a (Shimadzu XRD 600) diffract meter, XRD patterns between 2θ of 2° to 10° were obtained at a scan rate of 2°/ min on the diffract meter with CuK radiation a generator voltage of 40KV, and a generator current of 40mA and a wavelength of 0.1546nm at room temperature.

Cultivation of pathogenic strains

Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Micrococcus luteus, Bacillus cereus, Bacillus subtilis, Bacillus polymyxa, Bacillus thurgensis, salmonella Sp and Streptomyces Sp were cultured on broth flasks containing lysogeny broth medium and at 37°C for 24 h for investigation the antibacterial activity of both Ag/ PVA nanoparticles and nisin.

Preparation of nisin stock solution

A stock solution of nisin was purified from *Lactococcus lactis* Fc2 isolated and identified by chemical method previously cited by Abdel Karem *et al*. (2005). It was prepared by dissolving 0.5 g of nisin either standard or purified by chloroform in a 50 ml volumetric flask, 0.02 M HCl as diluents, was added then the mixture was boiled for 5 min. The stock solution was used to prepare nisin standards containing 0-1000 unit/mg powder using sterile 0.02M HCl as diluents (Wolf and Gibbons, 1995).

The test of organisms with nisin and Ag/ PVA nanocomposite

Bacterial susceptibility to antibiotics and nicin can be assessed using the agar diffusion method. In one implementation, 6 mm paper disks saturated with both nisin and Ag/ PVA nanocomposite solution at different concentrations are placed on the surface of an agar plate seeded with the test strain. For each of the eleven organisms mentioned in the experimental part, a set of standard bioassay plates (1.5% agar, 1% Tween 20, filter paper disks have 6mm diameter) as assay medium was prepared. From stock solution of nisin standard and purified 30µl was inoculated on sterilized filter paper discs comparing with different concentrations of antibiotics discs, replicated three times on each set of plates, following incubating the plates at suitable temperature for every indicator strain. The zones of inhibition were measured which resulted from both nisin and Ag/ PVA nanocomposite.

Results and Discussion

UV Spectroscopic analysis

The advantage of gamma irradiation method for the synthesis of metallic nanoparticles lies in the fact that desired highly reducing radicals can be generated without formation of any by-product. Fig. 1. show UV-visible

absorption spectra of pure PVA, regarding to this absorption spectrum, it can be seen that a nearly zero absorption in the wavelength range (600-300nm). But there is a strong absorption peak in the wavelength range (300-200nm). It is well known that PVA film is transparent and contains only single bonds; therefore it would be expected to absorb radiation only in the far UV region (120-200nm). The absorption band at 280nm may be assigned to π-π^* transition. This transition was related to the carbonyl groups (C=O) associated with ethylene instauration (C=C) of the type $(CH=CH)_2CO–$. The existence of carbonyl functionalities is probably due to residual acetate groups remaining after the manufacture of PVA from hydrolysis of poly vinyl acetate or oxidation during manufacturing and processing (Jayasekara *et al.*, 2004). When $AgNO_3$ precursor is embedded in PVA matrix, the optical properties are the net result of the electronic transition of the two materials. One can see that a strong increase in the absorbance in the visible regions i.e. in the spectral range 600-200nm, the absorption coefficient of the samples was increased with the increasing of the irradiation dose (Fig. 1).

The electronic spectrum of 25 kGy irradiated Ag/ PVA nanocomposite shows weak and broad absorption band in the visible region at 422nm. This band is assigned as plasmon resonance band of the small quantity of Ag-NPs formed at low irradiation dose (Whelan *et al.*, 2004). As the irradiation dose increased (from 25kGy to 100kGy), the plasmon band is shifted from 422 to 404nm (blue shift), indicating quantum size effect i.e. formation of smaller particles **(Weihong *et al.*, 2009)**.

The particle size is probably related to the amount of the stabilizing polymer's chains. With increasing irradiation dose, the individual macromolecules of PVA are assumed to be crosslinked with each other, giving rise to a three dimensional network. The crosslinking of polymer molecules results in a significant increase in molecular mass. This in turn will increase the amount of polymer chains surrounding the nanoparticles (Ravindrachary *et al.*, 2010). The more polymer chains there are, the more they inhibit the aggregation and/or the growth of the Ag-NPs. In addition, the increase of irradiation dose will increase the nucleation rate which results in the formation of smaller particles. The plasmon peak gets narrower and sharper and also increases in the intensity with increasing irradiation dose from 25 kGy to 100kGy.

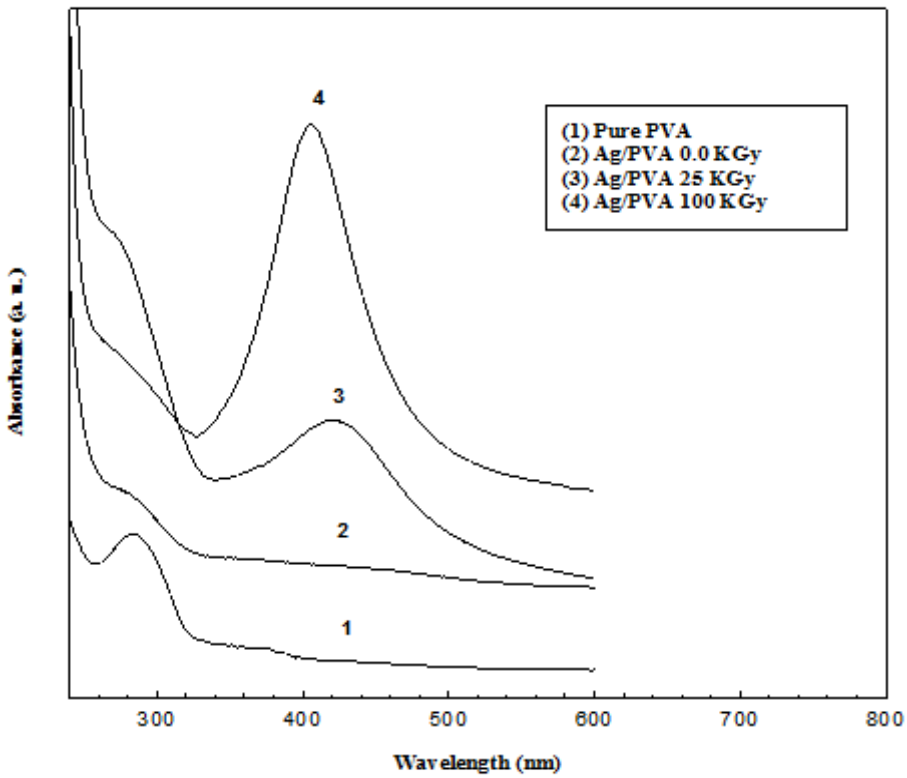

Fig.1 UV/ VIS spectra of pure PVA and irradiated Ag/ PVA nanocomposite.

XRD characterization

A typical XRD pattern, Fig. 2. displays the XRD patterns of pure PVA and Ag/ PVA nanocomposites irradiated with different gamma irradiation doses.

The XRD of pure PVA exhibit strong and broad diffraction peak located at $2\theta = 19.66^{\circ}$. The diffraction peak at $2\theta = 19.66^{\circ}$ corresponds to the (110) reflection, a plane which contains the extended planar zig-zag chain direction of the crystallites (Strawhecker and Manias, 2000). A more precise examination and inter comparison of diffraction patterns of different Ag/ PVA nanocomposites with that of pure PVA lead to three remarks. First, the as-prepared nanocomposites show four new diffraction peaks at $2\theta = 37.8$, 44.96, 64.34 and 77.8°, the discernible peaks can be indexed to the planes (111), (200), (220) and (311) respectively corresponds to face centre cubic structure of silver. Second, there is a broadening as the irradiation dose increases from 25kGy to 100kGy

for Ag lines in accordance with their small grain sizes. These nanocrystals have lesser lattice planes compared to bulk, which contributes to the broadening of the peaks in the diffraction pattern. It is well known that the average particle size of spherical crystallites can be determined by measuring the full wave at half maximum, (FWHM), of the main diffraction peaks. The particle size (D) was calculated based on the regular broadening of XRD peaks as a function of decreasing crystallite size.

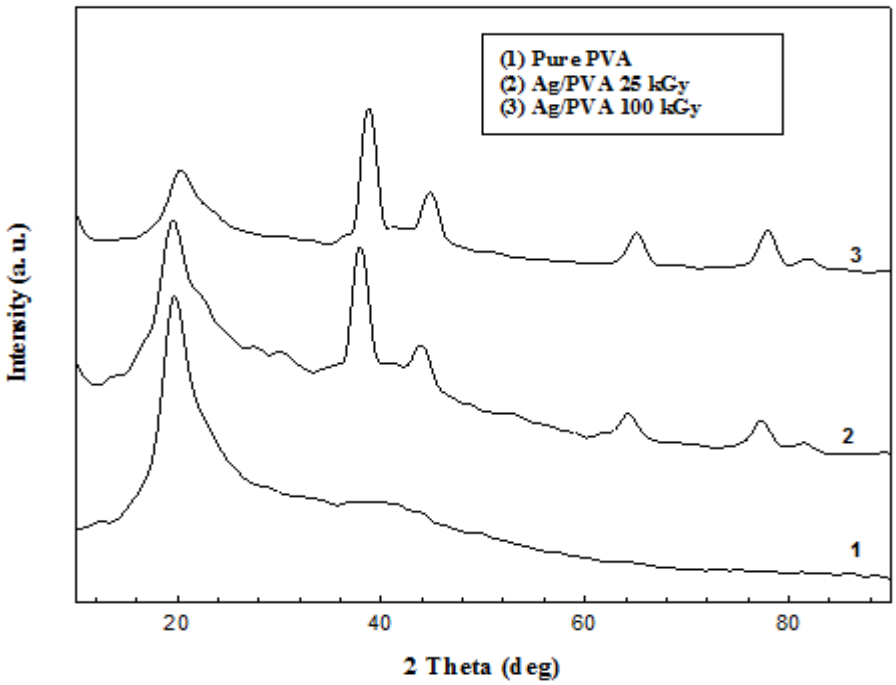

Fig. 2. XRD pattern for pure PVA and irradiated Ag/ PVA nanocomposites.

This broadening is a fundamental property of XRD described by well-established Scherer theory, equation (1): $D = k\lambda/ \beta \cos\theta$ (1)

where, D is the particle diameter (nm), K is a constant equals 0.9, λ is the X-ray wavelength; (CuKά = 1.540˚ A), β is FWHM of the peak corresponding to plane (200) and θ is the diffraction angle equals. The calculated particle size was 4nm for 25kGy irradiated sample and 2nm for 100kGy. These confirm the effect of the irradiation doses on the particle size (*i.e.* the particle size decrease with increasing the irradiation dose).

TEM characterization

Controlling of the size, morphology and distribution of nanoparticles plays an important role in the properties of nanocomposites. Thus to get a real idea about what is happening inside the matrix system, TEM micrographs was taken into account.

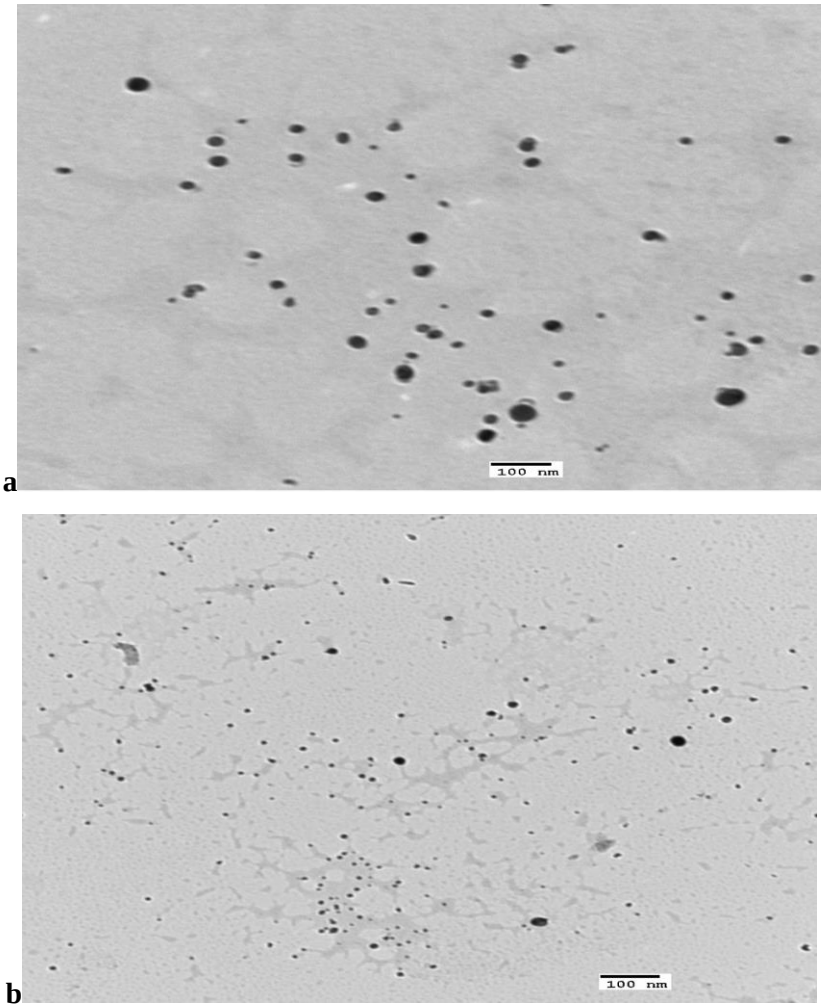

Fig. 3. TEM image of irradiated PVA/ Ag nanocomposite, (a) 25kGy (b) 100kGy.

Fig. 3a. represents TEM images of Ag nanoparticles prepared in PVA matrix with 25kGy irradiation dose. A number of well-dispersed nanoparticles

can clearly be seen in the TEM picture with external spherical shape and are to a large extent well-separated from one another and the average diameter of the particles is indicated to be 23nm. The Ag nanoparticles prepared in the PVA under gamma irradiation (100kGy) is given in Fig. 3b. Regarding to the image, the particles exhibit a uniform shape and a narrow size distribution with average size of 7nm. This result means that, the size of the prepared particles gets smaller and the particle size distribution is improved with increasing the irradiation dose. There is a mismatch between the values of the grain size calculated from XRD and TEM. It should be pointed out that each method examines somewhat different aspects of the particle size. So it not surprising that the reported grain size of Ag nanoparticles very widely between measurements methods.

Antibacterial characterization

Ag-NPs mode of action

The mechanism for anti-bacterial action of Ag-NPs is bacterial membrane disruption by the ions silver released from the PVA. The Ag+ ions form insoluble compounds with sulphydryls groups in the cellular wall of the micro organism that are responsible for the inhibition halo in the seeded culture media. This result can be explained in terms of the presence of hydroxyl groups in the PVA chain and it is easy to induce Ag+ motility. The Ag+ release mechanism is not elucidated. However, it is possible that the hydroxyl group improves the Ag+/H+ ionic exchange (Babak Sadeghi *et al.,* 2012). The antibacterial properties of the nanocomposites were tested against some gram-positive *S. aureus* and some gram-negative *E. coli*. Table 1, shows typical antibacterial test results of Ag/ PVA nanocomposites against *S. aureus* and *E. coli* determined by a disk method. Antibacterial ability, measured by the diameter of the growth inhibition zone, depended on the test sample used. Ag-NPs are harmful to bacteria according to Chamakura *et al.* (2011), it was found that Ag-NPs react with cell walls and cytoplasmic membranes of *Escherichia coli*, resulting in pits in the cell wall of bacteria, and finally killing them. Last results demonstrated that the irradiated Ag/ PVA nanocomposites showed greater antibacterial effect than un-irradiated nisin antibiotic, this effect increase with increasing irradiation dose. These results in a good agreement with the XRD and TEM which confirm the decreasing in the particle size with increasing the irradiation dose which leading to a good antibacterial activity.

TABLE 1. Radius inhibition zones (RIZ) of control Ag/ PVA and irradiated Ag/ PVA as compared with nisin.

Indicator strain	Dose (kGy)	RIZ of (Ag/ PVA)	RIZ of nisin
Listeria monocytogenes	0.0	1.3	1.4
	25	1.8	--
	100	2.1	--
Pseudomonas aeruginosa	0.0	2.9	2.4
	25	3.1	--
	100	3.4	--
Staphylococcus aureu	0.0	0.8	0.7
	25	1.0	--
	100	1.4	--
Bacillus subtilis	0.0	2.6	2.2
	25	2.8	--
	100	3.0	--
Bacillus cereus	0.0	2.4	1.9
	25	2.5	--
	100	2.7	--
Escherichia coli	0.0	1.7	1.6
	25	1.9	--
	100	2.2	--
Bacillus thurgensis	0.0	0.6	0.4
	25	0.8	--
	100	0.9	--
***salmonella* Sp**	0.0	0.7	0.3
	25	0.8	--
	100	1.0	--
Micrococcus luteus	0.0	2.0	1.6
	25	2.7	--
	100	2.9	--
Bacillus polymyxa	0.0	2.6	2.3
	25	3.0	--
	100	3.2	--

Nisin mode of action

The interference of the polypeptide antibiotic nisin with membrane function and murein synthesis was studied, the inhibition of the *in vivo* synthesis of murein in *Bacillus subtilis* and the resistance of mycoplasma miscodes to nisin, showed the membrane may be excluded as a target. On growth inhibition by nisin with both, cytoplasmic membranes and isolated phospholipid components of membranes were demonstrated. The lyses of phospholipids liposome by nisin and sensitivity at increased concentration of

antibiotic of organisms lacking murein in their cell walls provide further evidence for the cytoplasmic membrane as a target (Ten Brink *et al.*, 1994). Hammes *et al*. (1986) reported that at a higher concentration, nisin can inhibit peptidoglycan synthesis. In cell free systems, nisin caused the accumulation of undecaprenyl-pyrophosphomurein in N (lipid intermediate)**.** The accumulation was caused by a complex formation between nisin and lipid intermediate (Reisinger *et al.*, 1980), according to the following equation:

UDP-MurNAc (pentapeptide)+ C_{55}P translocase C_{55}-PP-MurNAc (pentapeptide)+ UMP. (2)

UDP-GlcNAc+ C_{55}-PP-MurNAc (pentapeptide) transglycosylase UDP+ C_{55}-PP-MurNAc (pentapeptide)-GlcNAc. (3)

where, MurNAc is N-acetyl muramic acid, GlcNAc is N-acetyl glucose and

UPD is uridine diphosphate.

In the presence of nisin, reaction (2) is activated, whereas reaction (3) is inhibited. Nisin does not need any receptors, unlike some other antimicrobial peptides, it does need the presence of a membrane potential (Bruno *et al.*, 1992). The dehydro-amino acids have been suggested to interact with sulfhydryl groups of enzymes. Jack *et al*. (1994) mentioned that nisin interferes with the energy supply of the cell. Pores are thought to be created in the cell membrane allowing dissipation of the membrane potential. Cell lyses has been explained by a cationic exchange like process, where the strongly cationic lantibiotics displace autolytic enzymes. The enzymes weaken the cell wall. The lantibiotics interfere with the cells energy supply, inhibiting cell wall repair. The pores formed by the lantibiotics do not allow passage of high molecular wt compounds, resulting in net influx of water increasing the osmotic pressure and causing cell lyses (Davidson and Hoover, 1993).

Conclusion

In summary, the synthesis of Ag/ PVA nanocomposites by a simple and easy route was described in the presence of PVA as a stabilizer. The prepared Ag nanocomposites have a smaller grain size and more narrow grain distribution and PVA can efficiently protect the nanoparticles from aggregation. The Ag nanocomposites have a very good antibacterial activity towards both Gram-positive and gram-negative bacteria compared with nisin produced by *lactococcus lactis*, which increase by increasing irradiation dose.

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(Received: 23/09/2012;

accepted: 08/11/2012)

تحضير وتوصيف متراكبات نانومتريه من الفضه والبولى فينيل الكحول كمضادات للبكتريا

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قسم الكيمياء الاشعاعية ، المركز القومى لبحوث وتكنولوجيا الاشعاع ، ص. ب. ٢٩ مدينة نصر ، القاهرة ، مصر و *قسم الكيمياء ، كلية العلوم ، جامعة عين شمس ، القاهرة ، مصر.

يهدف البحث الى تحضير وتوصيف متراكبات نانومتريه من:- (Ag/ PVA) ومقارنتها كمضادات للبكتريا بالبكتريوسين (النيسين) والتى تشتمل تطبقاتهما على منتجات حماية الاسنان والمركبات الصيدلانيه.

وقد تم تحضير المتراكبات النانومتريه من (Ag/PVA) بطريقة الاختزال الذاتى فى وجود الاشعاع الجامى. وكانت المتراكبات المتحصل عليها لها نشاط مضاد للبكتريا (جرام الموجبه والسالبه).

وقد تم اجراء دراسات اخرى توضح التركيب والتوزيع الكيميائى لجزيئات الفضه فى بوليمر كحول البولى فينيل مثل جهاز حيود الاشعه السينيه و الميكروسكوب الاكترونى النافذ.