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Original Article

Physicochemical Characterization and Antimicrobial Activity of Nanosilver Containing Hydrogels

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Abstract

Silver ion has been used for centuries to prevent and treat a variety of diseases and infections. In recent years, extensive studies have been undertaken on the use of antimicrobial properties of silver, incorporated within medical devices. The aim of this study was to prepare a formulation containing silver ion, which could be applied for wound dressing. The purity of nanosilver was measured by atomic absorption spectrophotometer. The purity was determined to be 96.80 % ±0.01. The "serial dilution method" was used to determine the Minimum Inhibitory Concentration (MIC) of nanosilver, on microorganisms such as *staphylococcus aureus*, and *Pseudomonas aeruginosa*. The MIC of nanosilver solution was 15.12 µg/ml. In order to design a hydrogel formulation, different formulations, using HPMC K15M, were made. Next, the best formulation (at 2% w/w) was selected, based on attractiveness, homogeneity and flexibility. This formulation was used for the preparation of the next formulations, containing various percentages of two types of plasticizer. Again, the best formulation was chosen. In order to increase the thickness and resistance of the film, another polymer (HPMC K100 or agar), was added to the formulation. For evaluation of the prepared films, different tests including determination of thickness and tensile strength, swelling and water vapor transition were performed. Finally, the best formulation containing 2% w/w HPMC K15M, 0.5% w/w HPMC K100 and 0.2% triacetin (as plasticizer)was selected. Then, various concentrations of nanosilver solution were added to the selected formulation. In this manner, the most suitable concentration of nanosilver (4 mg in an area of 100 cm^2 or almost 1.5 g weight), which had the best antimicrobial effect in the hydrogel films was detected. In the final stage, the amount of silver in the final film was determined by atomic absorption spectrophotometer. The obtained result confirmed the amount of silver in the final film.

Keywords: Hydrogel; HPMC; Nanosilver; Ag-NPs; MIC.

Introduction

Research has been intensive on antibacterial materials containing various natural and inorganic substances (1). Among them, silver or silver ions have long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities (2). It is surprising that these applications were used without understanding the mechanism of action of silver ion. For example, foods were kept in silver plates, since it has been found that it is more stable. Also, silver ion has been used for the treatment and prevention of injured soldiers in India (3). Recent microbiological

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and chemical experiments implied that the interaction of silver ion with thiol groups, plays an essential role in bacterial inactivation (4). Nowadays, the antimicrobial properties of silver are used in so many cases and its mechanism is known as inactivation the cellular metabolism or restraining the growth of cells. Silver weakens breathing of the bacterial cell and affects the circle of transference of electrons and also deranges the transit of materials from bacterial membrane (5). Possessing high specific surface area and a high fraction of surface atoms, metal nanosilver has been studied extensively due to it's unique physicochemical characteristics, such as catalytic and antimicrobial activity, optical, electronic and magnetic properties (6). It can be expected that the presence of a high specific surface area and fraction of surface atoms of nanosilver, could lead to high antimicrobial activity compared to bulk silver metal.

Silver does not interfere with the medical properties of drugs and it is being widely used. There are four major methods to use silver for antimicrobial purposes: traditional coating method with silver, slight absorption of silver in hydrogel bases, mixing silver with other materials and the use of nanoparticles (7).

Also, there are two necessary conditions to achieve the maximum antimicrobial effect of silver, namely a large surface area of silver (nanoparticle) and presence of a sufficient amount of moisture in hydrogel. Adding silver in nano- form to polymerich hydrogel systems, provides both of these conditions for more effectiveness of silver (8). The aim of this study was to prepare a formulation containing silver ion, which could be applied for wound dressing. The other purpose was to examine the antibacterial activity of silver nanoparticles (Ag-NPs) against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Experimental

Materials

Nanosilver (Ag-NPs) was obtained as a donation from (Nano-naseb) Iran; Hydroxypropylmethylcellulose (Methocel K15 M, and Methocel K 100) was obtained as a gift from Colorcon Ltd. (U.K.); Triacetin, Dibutyl phthalate and Polyvinyl pyrollidone (PVP) were obtained from Merck, Germany. Culture media (Triptone soy agar from Pronadisa, Spain), were also used in this study.

Methods

Identification and determination of purity of Nanosilver

Identification and determination of nanosilver was performed with an atomic absorption instrument (Anal.Tech., England) using standard curve of silver nitrate (9).

Preparation of hydrogel base

Gels (100 g portions) were prepared by heating two thirds of the total amount of freshly prepared distilled water to 80 °C and then adding the required amount of polymer for dispersion. Following the addition of the required amount of plasticizer, the gels were thoroughly mixed. Cold water was then added to bring gels up to weight. The gels were triturated for 1 h to a uniform consistency and left overnight to equilibrate. Gels were centrifuged at 500 rpm for 20 min, and were then poured into special glass vessels (10 x 10 cm^2). The gels were dried at 55 °C in an oven for 24 h (10).

Preparation of hydrogel containing one type of polymer

Gels containing 1, 2, 3, 4 and 5% w/w of HPMC K15 M; 5, 8 and 10% w/w of HPMC K 100 and 1, 3 and 5% w/w of PVP 700000 with 0.2% w/w of either dibutyl phthalate or triacetin, as plasticizer, were made. Then, the gels were evaluated from the appearance point of view. The best formulation, among the prepared gels, was chosen and new formulations with different concentrations of plasticizers (0.4, 0.6, 0.8, 1 and 1.5% w/w based on polymer content) were prepared. The prepared gels were examined from the point of view of flexibility and the best formulation was chosen in the next step.

Preparation of hydrogels containing two types of polymers

Different amounts (0.5, 1 and 2% w/w) of either Agar or HPMC K 100 were added to each formulation. The prepared formulations were compared from the point of view of their

appearance. Then, new formulations with different concentrations of plasticizers (0.4, and 0.8 w/w based on polymer content) were made. The prepared gels were examined from the point of view of flexibility and the best formulation was chosen.

Investigation of physicochemical properties of the prepared films

Determination of film thickness

Thickness of the prepared film was determined with a digital caliper. To accomplish this, thickness of 5 different parts of each film was measured and the mean was determined as the film thickness.

Water vapor transition test

A cylindrical dish with an internal diameter of 3.5 cm and a height of 2 cm was used. First, one cell was filled with 10 ml of distilled water, then covered with the prepared film completely, next weighed by an analytical balance, and finally placed in a desiccator containing calcium chloride. Another dish was chosen as th control, containing no water, and it's edge was covered with the film. After weighing, it was put in a desiccator. At different time intervals, (24, 48, 72 and 96 h), both dishes were taken out of the desiccator and re-weighed (±0.00001). At the end of the test, a curve of weight changes against time was drawn. The amount of water vapor transition from the prepared film was measured, using equation 1 (11).

$$WVT = \frac{Wx}{tAP_0(RH_1 - RH_2)} \qquad \text{(Equation 1)}$$

In this equation, WVT is the water vapor transition.

W/t is the mass change (flux, g/h) resulted from the slope of profile of the mass change versus time, x is the thickness of the film used in the scale of mm. A is the area of the film in the scale of m^2, which is equal to the surface of the dish. P_0 is the vapor pressure of pure water vapor transition that is equal to 3.159 kpa at 25 °C. (RH_1-RH_2) is the relative humidity gradient of the inside and outside moisture contact of the examined dish.

Swelling test

The effect of different concentrations of plasticizer on the swelling indicator of the prepared films was studied. Dried films (1x1cm) were cut and submerged in water for 15 min. Then, the film was brought out, its surface was dried and weighed on a analytical balance. This process was repeated till the film was scattered. The indicator of the swelling was calculated, using equation 2.

$$I_s(\%) = \frac{Ws - Wd}{Wd} \times 100 \qquad \text{(Equation 2)}$$

In here, I_s, is the swelling indicator (%), W_d, is the weight of dried polymer film before being immersed in water and W_s, is the weight of film after swelling (11).

Determination of tensile strength

Tensile strength was determined by the Zwick device. Prepared films (1×5cm) containing different concentrations of both kinds of plasticizers, Triacetin and Dibutyl phthalate, were cut and tested. First, the film was connected to the lower and the upper jaws of the device, then, one of the jaws was fixed and the other was moved at a speed of 10 mm per minute. This device determines the tensile strength and resistance of the film to the tearing point, based on the desired units (12).

Determination of the Minimum Inhibitory Concentration (MIC)

To determine the MIC of the silver, the serial dilution method was used. Each of the twelve test tubes was filled with 1ml of the liquid nutrient broth medium. Into each of the test tubes number 1 and 2, one ml solution containing 2000 µg/ml of silver was added and mixed thoroughly with the culture medium. In this case, the concentration of silver in each test tube was 1000 µg/ml.

Then, 1 ml of the content of test tube number 2 was added to test tube number 3 and mixed completely. This process was performed serially to test tube number 11. At the end, 1 ml content of test tube number 11 was discarded. Eventually, 0.1 ml of standard microbial suspensions (*Staphylococcus aureus* or *Pseudomonas*

aeruginosa containing 10^8 microorganism per ml), were added to test tubes number 2 to 12, and in order to have equal amounts of material in all the test tubes, 0.9 ml of test tube number 1 was discarded. The test tubes were incubated at 32-35 °C for 24 h. Then, the microbial growth was studied by turbidimetric measurement, using a spectrophotometer (13).

Addition of nanosilver to hydrogel bases

As mentioned previously (preparation of hydrogel base), suitable amounts of polymer was dispersed in water and after adding the plasticizer, different amounts of nanosilver were added to the bases and at the end, the final weight of each base was adjusted by the addition of distilled water. The prepared suspension was blended for 1 h with mechanical blender, and centrifuged at 500 rpm for 20 min. It was then placed in a refrigerator for 24 h, for the emergence of bubbles and polymer swelling. The final gel was poured into a glass mould and placed in an oven at 55 °C for 24 h. At the end, the films with different proportions of nanosilver (8, 4 and 2 mg/100 cm area) were examined for determination of the best silver content, resulting in a suitable antimicrobial effect in the prepared films (14, 15).

Anti-microbial adequacy test

To determine the anti-microbial adequacy of the prepared films containing nanosilver, 100 µl of standard microbial suspensions of *Staphylococcus aureus* and *Pseudomonas aerugninosa*, were spread on the culture medium (triptone soy agar, TSA). Films (5×5 cm) containing silver (bandage) were cut and placed on the culture medium. In the other group no bandage was used. All the plates were placed at 25±3 °C for 24 h and then studied for bacterial growth (14).

Results and Discussion

Determination of purity of nanosilver

In order to determine the purity of nanosilver, solutions containing various concentrations of standard silver nitrate were prepared, and their absorption values were measured, using an atomic absorption spectrophotometer. The obtained data were used for plotting the standard curve. Next, the absorption of the sample solutions containing nanosilver were determined. By using the standard curve, the purity of the sample solutions were found to be 96.80 % ±0.01.

Choosing the best hydrogel base containing one type of polymer

All the prepared formulations were compared to each other, based on their visual properties (such as the amount of bubble), flexibility, and homogeneity in the dried gel. These properties were studied in order to choose the best gel, with the most acceptable appearance. Furthermore, a change in the percentage of various polymers or plasticizers, separately, justifies the influence of various ingredients on the final appearance of the hydrogel base.

Because of the high viscosity of the polymer HPMC K15M, the 5% polymer gel had a high visosity, in presence of each type of plasticizer. Hence the formulation containing 2% w/w of HPMC K15M, with 0.2% of plasticizer (triacetin or dibutyl phthalate), was chosen due of it's suitable viscosity and ease of transformance. In addition, after drying, it was separated easily form the bar and found to be flexible.

It's necessary to mention that a reduction of percentage in the polymer in the presence of a fixed percentage of plasticizer leads to an increase in the percentage of plasticizer, compared to the polymer, and with entering more plasticizer molecules between the polymer molecules, the flexibility of the film increases drastically. Furthermore, a reduction in viscosity leads to an easier emergence of bubbles, making the appearance of the product more attractive with less bubble.

In formulations made by the polymer HPMC K100, which is a polymer with a low viscosity grade, the final gel was thin and the polymer film was very narrow and fragile.

Since PVP has been used in wound healing by other researchers (8), it was selected for further investigation and the preparation of the film. But since PVP has a high viscosity, formulations made from it (even in 1% w/w) were too viscous and the obtained film was fragile, with no flexibility. Therefore, PVP was

omitted from this study.

In addition, for improving the flexibility of the prepared films, the best formulations were chosen and prepared with different percentages of plasticizer, in order to make new formulations. Formulations containing 2% HPMC K15M and 0.8% dibutyl phthalate or 1% triacetin had the best flexibility and the prepared film did not break on bending. Furthermore, no sign of bending was observed on the film.

Choosing the best hydrogel base containing two kinds of polymer

Since a natural polymer has been used in the formulations of dressings like Aqua-gel® and KIK-gel® (16) and the resulting films reported to have high mechanical and elastic properties, therefore, agar was added to the best formulation obtained from the previous stage.

Addition of agar to the hydrogel base separated the phases of the gels and none of the plasticizers created a desirable formulation, from the point of view of appearance. Hence, a polymer with a low viscosity grade (HPMC K100) was chosen for inclusion within the hydrogel base, for further studies. The reason for choosing this polymer was based on some reports (10), stating that mixing two different cellulose polymers could have a synergistic effect on the viscosity and could improve their characteristics.

Addition of 0.5, 1, and 2% w/w of HPMC K100 to the hydrogel base containing 2% w/w of HPMC K15M was investigated. It was observed that in the case of 0.5% w/w of HPMC K100, the homogeneity and thickness of the final film increased to the desired level and an appropriate film flexibility was observed.

The effect of plasticizers on film thickness

When the amount of plasticizer (triacetin or dibutyl phthalate) in the new formulations increased from 8 to 32% w/w, based on the polymer content, the film thickness increased from 0.051 to 0.110 (for triacetin), and 0.071 to 0.119 (for dibutyl phthalate), respectively. The thickness of the films made by the plasticizer dibutyl phthalate is greater than the films made by triacetin. This difference can be related to the higher molecular weight of dibutyl phthalate. Settling of the dibutyl phthalate molecules between the HPMC polymeric chains, could be a reason for the greater thickness of films containing this plasticizer (17).

The effect of plasticizers on water vapor transition from prepared films

Table 1 shows the effect of different concentrations of plasticizers on water vapor transition from the prepared formulations. It was found that an, increase in the percentage of both types of plasticizers, raises the amount of water vapor transition and this is possibly because both plasticizers are hydrophilic (17). The plasticizer molecules present within the polymer particles absorb water, and on the other hand due to the presence of moisture absorbing materials in the desiccator, the water molecules come out of the other side of the film and are absorbed by the water absorbing material. Presence of a higher percentage of plasticizer between the polymer particles, makes the above mentioned process faster and the amount of water vapor transition would be greater. Since the goal of producing these films is to make dressing for wounds and considering the accomplished experiments, keeping the moisture on the surface of the injury

Table 1. The effect of plasticizer content on water vapor transition (WVT) from the prepared films containing 2% w/w HPMC K15M,and 0.5% HPMC K100 (n=3).

Type of plasticizer	Amount of plasticizer based on polymer content (%)	Weight before drying(g)	Mean thickness (mm)	WVT X10^4	WVT x10^4 / mean thickness (mm)
Dibutyl phthalate	8	30	0.051	1.936	37.975
	16	30	0.054	2.089	38.154
	32	40	0.063	3.816	45.975
Triacetin	8	30	0.069	2.328	33.739
	16	30	0.069	2.936	42.558
	32	40	0.078	3.994	50.771

Table 2. The effect of plasticizer content on swelling indicator of the prepared films containing 2% w/w HPMC K15M, and 0.5% HPMC K100 (n=3).

Type of plasticizer	Amount of plasticizer based on polymer content (%)	Mean W_d(mg)	SD X10²	Mean W_s(mg)	SD X10²	I_s
Dibutyl phthalate	8	7.2	4	34.7	1	388.212
	16	8.1	1	47.5	2	482.215
	32	10.2	0.9	63.5	2	522.217
Triacetin	8	6.5	1	43.3	2	558.937
	16	6.9	0.6	44.9	0.4	551.788
	32	7.1	1	48.1	2	575.443

Wd: Weight of dried polymer befor being immersed in water; SD: Standard Deviation; Ws: Weight of film after swelling; I_s: Swelling indicator.

increases the speed of healing. As a result, the ideal formulations for this goal are those with less water vapor transition, which can also maintain more moisture on the surface of the wound. Based on the results, films containing 8% w/w of plasticizers (based on polymer content) are more suitable for this goal.

The effect of plasticizers on swelling indicator of the prepared films

Table 2 shows the effect of different percentages of both types of plasticizers on the chosen formulations. It was noted that as the concentration of dibutyl phthalate increased, the swelling indicator also increased. This increase in polymeric films containing dibutyl phthalate shows a significant difference, in comparison with triacetin. On the other hand, since the swelling indicator in films containing triacetin is generally higher, the swelling indicator at a concentration of 8% w/w, is more than the swelling indicator at a dibutyl phthalate concentration of 32%. It is possible that the greater hydrophilicity and water absorption ability of triacetin could help to scatters the film so fast, that is not desirable (17).

The effect of plasticizers on tensile strength of prepared films

As could be seen in Table 3, with increasing the percentage of both types of plasticizers, the tensile strength of the prepared formulation will decrease. A greater decrease in tensile strength in formulations with triacetin is observed. Also, at a concentration of 8% w/w, of both plasticizers, the greatest resistance was observed. In the case of polymeric films made from HPMC, the most effective factor is linkage between monomers, since by increasing the percentage of plasticizer and their settlement between the polymer particles, the distance between the monomers will increase and this could reduce the linkage between monomers, leading to a reduction in tensile strength. Since increasing the dibutyl phthalate concentration has a lower effect on the tensile strength of the polymeric film, possibly by combining different percentages of both types of plasticizers in a polymeric film, a desirable

Table 3. The effect of plasticizers content on tensile strength of prepared film containing 2% w/w HPMC K15M, 0.5% HPMC K100 (n=3).

Type of plasticizer	Plasticizer based on polymer content (%)	Mean Tensile strength	SD
Dibutyl phthalate	8	53.134	2.16
	16	45.597	0.38
	32	44.281	2.38
Triacetin	8	116.398	1.18
	16	56.114	0.98
	32	26.582	1.88

SD: Standard Deviation

percentage could be reached, giving a suitable flexibility, with a high tensile strength.

Determination of MIC of nanosilver

As mentioned in the determination of MIC for nanosilver, 12 test tubes were used, containing a gradually reducing nanosilver content from 2000 µg/ml to less than 2 µg/ml. The MIC is the concentration of solution in a test tube with a concentration lower than that which could prevent the growth of microorganisms, and hence the solution remains clear. Hence, the first tube that changed turbidity, indicates the growth of microorganisms. The MIC was observed to be 15.625 µg/ml. Since the purity of nanosilver was 96.8%, therefore, the MIC of nanosilver solution was determined to be 15.12 µg/ml.

The MIC of silver was reported to be 7.5 µg/ml for *pesuodomonas aueruginosa* and 12.5 µg /ml for *staphylococcus aureus*, respectively (2). Feng *et al*, (1) reported an MIC of 14.1 mg and 28.1 mg/1 of silver for *Escherichia coli* and *Candida albicans,* respectively. Results of the microbial adequacy test, in plates containing films with 4000 mg of nanosilver in 100 cm, showed no microbial growth .

Adequacy of the prepared films

Some silver formulations that now are being used worldwide, contain different proportions of silver in 100 cm^2, include Aquacel-Ag®, Silvasorb® and Actisorb sivler 220® containing 8.3, 5.3 and 2.7 mg silver in 100 cm^2, respectively (18). The produced formulation contained 4 mg of silver in 100 cm^2, which had the power to restrain the microbial growth. Regarding the mentioned points, the suggested formulation contains 2% HPMC K15M, 0.5% HPMC K100, 0.2% of the plasticizer dibutyl phthalate and 4 mg of nanosilver.

Conclusions

In this study the "serial dilution method" was used to determine the MIC of nanosilver, on microorganisms such as *staphylococcus aureus*, and *Pseudomonas aeruginosa*. The MIC of nanosilver solution was 15.12 µg/ml. Hydrogels containing nanosilver were prepared. For evaluation of the prepared films, different tests, including the determination of thickness and tensile strength, swelling and water vapor transition were performed. The finally chosen formulation contained 2% w/w HPMC K15M, 0.5% w/w HPMC K100 and 0.2% triacetin (as plasticizer). Various concentrations of nanosilver solutions were added to the selected formulation. The ultimate concentration of nanosilver (4 mg in an area of 100 cm^2 or almost 1.5 g weight), which had the best antimicrobial effect in the hydrogel films was detected.

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