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Preparation and *in vitro* Bactericidal and Fungicidal Efficiency of NanoSilver / Methylcellulose Hydrogel

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***Abstract*—**In this work we describe the preparation of NanoSilver/methylcellulose hydrogel containing silver nanoparticles (NPs) for topical bactericidal applications. Highly concentrated dispersion of silver NPs as high as of 5g/L of silver with diameter of 10nm was prepared by reduction of $AgNO_3$ via strong reducing agent $NaBH_4$. Silver NPs were stabilized by addition of sodium polyacrylate in order to prevent their aggregation at such high concentration. This way synthesized silver NPs were subsequently incorporated into methylcellulose suspension at elevated temperature resulting in formation of NanoSilver/methylcellulose hydrogel when temperature cooled down to laboratory conditions. *In vitro* antibacterial activity assay proved high bactericidal and fungicidal efficiency of silver NPs alone in the form of dispersion as well as in the form of hydrogel against broad spectrum of bacteria and yeasts including highly multiresistant strains such as methicillin-resistant *Staphylococcus aureus*. A very low concentrations of silver as low as 0.84mg/L Ag in as-prepared dispersion gave antibacterial performance. NanoSilver/methylcellulose hydrogel showed antibacterial action at the lowest used silver concentration equal to 25mg/L. Such prepared NanoSilver/methylcellulose hydrogel represent promising topical antimicrobial formulation for treatment of burns and wounds.

***Keywords*—**Antimicrobial, burn, hydrogel, silver NPs.

I. Introduction

SILVER NPs are among the promising nanomaterials having great application potential in medicine due to their high antimicrobial efficiency. Antimicrobial effects of silver NPs are intensively studied particularly in recent years when classical antibiotic therapy is limited by development and spread of bacterial resistance resulting in higher morbidity and mortality rates in patients with infections caused by multi-resistant bacteria [1], [2]. Silver NPs represent effective antimicrobial agent with ability to kill bacteria at such low concentrations in units of milligrams per litter [3]–[9] which do not show cytotoxic effect to mammalian cells [7], [10]. Moreover, no relevant data dealing with development of bacterial resistance to silver NPs were published in scientific literature. The reason why bacteria could not develop resistance to silver NPs is probably multimode of action of silver NPs at several levels of bacterial cell in comparison with specific and targeted mechanism of classical antibiotics. Silver NPs influence many bacterial structures and metabolic processes at the same time. Silver nanoparticles (AgNPs) were shown to inactivate bacterial enzymes [11], [12], disrupt bacterial metabolic processes [13], [14] and the bacterial cell wall, accumulate in the cytoplasmic membrane and increase its permeability [12], [3], [4], interact with DNA [11] and generate reactive oxygen species [15]–[17].

Burn injury affects natural skin barrier and some of the systemic host defense mechanisms resulting in microbial colonization in burn wound and eventually development of burn wound sepsis. Therefore topical antimicrobial treatment to control colonization and proliferation of microbial pathogens is the most important approach in burn wound care. Overuse and irrational application of antibiotics have contributed to development of bacterial resistance resulting in high mortality and morbidity of patients with burn wound. Regarding to this, silver based antimicrobial agents (e.g., silver nitrate and silver sulfadiazine) proved to be effective in burn wound care and significantly reduced morbidity and mortality. Thus Silver sulfadiazine (SSD) has become one of the leading topical antimicrobial agents used to treat burn wound infections over the last four decades [18]. Burn wound treatment using silver based compounds has many benefits such as multilevel antibacterial effect on bacteria which significantly reduce ability of bacteria to develop bacterial resistance. Moreover, silver is effective against highly resistant bacteria such as *Staphylococci* sp., *Enterococci* sp. etc. However, action of ionic silver (Ag^+) based antimicrobial agents (silver nitrate, silver sulfadiazine) used for topical applications may be limited by some adverse effects such as bonding of ionic silver with halogen anions or proteins in body fluids or gray skin discoloration (argyria) upon prolonged use, or dermal toxicity [19], [20]. On contrary, these limitations and adverse effects can be overcome when silver NPs are used instead of ionic silver. Silver NPs are as well effective against bacteria as ionic silver is and in addition silver NPs show much more less toxicity to human cells than ionic silver. Therefore, the combination of a hydrogel system with silver NPs would be a better choice for topical treatment of burn wounds [21]–[25].

In recent years, the hydrogels as well as water-swollen polymeric materials were developed due to their superior properties that include three-dimensional structure, hydrophilicity and water uptake capacity. Owing to hydrogel's unique properties, they are being used in treatment of burn wounds allowing good hydration which is important for quick healing and reepithelialization of the wound. However, a distinctive disadvantage of the commercially available

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hydrogels formulations and materials is that they do not provide a barrier against wound infection. Incorporation of silver NPs into hydrogels can overcome this disadvantage and provided effective bacterial barrier protecting bacterial penetration and colonization of burn wounds finally resulting in better and rapid wound or burn healing without complications connected with microbial infections.

In this work we describe the preparation of NanoSilver/methylcellulose hydrogel containing silver nanoparticles (NPs) for topical bactericidal applications. Stable and highly concentrated dispersion of silver NPs as high as of 5g/L of silver with diameter of 10nm was used for NanoSilver/methylcellulose hydrogel preparation. Both dispersion of silver NPs as well as NanoSilver/methylcellulose hydrogel proved to be effective against broad spectrum of bacteria and yeasts including highly multiresistant strains such as methicillin-resistant *Staphylococcus aureus*. Such prepared NanoSilver/Methylcellulose hydrogel represent promising topical antimicrobial formulation for treatment of burns and wounds.

II. Experimental Part

A. Chemicals and Biological Materials

For the synthesis of silver NPs silver nitrate (99.9%, Tamda), ammonia (p.a., 28% aqueous solution, Sigma-Aldrich), sodium hydroxide (p.a., Sigma-Aldrich), sodium borohydride (98.5, Sigma-Aldrich), sodium salt of polyacrylic acid (MW 1200, 45% aqueous solution, Sigma-Aldrich) were used. Methylcellulose (MW 40000, powder, viscosity 400 cP, Sigma-Aldrich) was used as hydrogel forming agent. All chemicals were used as-received without further purification.

For antimicrobial assays following bacterial strains, obtained from the Czech Collection of Microorganisms, Czech Republic (Masaryk University in Brno, Czech Republic), were used: *Staphylococcus aureus* CCM 3953, *Escherichia coli* CCM 3954 and *Pseudomonas aeruginosa* CCM 3955. For testing purposes, we also used bacterial strains isolated from clinical material of the University Hospital in Olomouc, Czech Republic. These strains included *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* 879, *Staphylococcus epidermidis* 901, methicilline-resistant *Staphylococcus aureus* (MRSA), and ESBL-positive *Klebsiella pneumoniae* 2486. Antimycotic activity was tested using *Candida albicans* (I and II), *Candida tropicalis* and *Candida parapsilosis* strains isolated from the blood of patients of the University Hospital in Olomouc, Czech Republic, who had confirmed candida sepsis. The yeasts were identified using conventional mycological procedures: (i) appearance on CHROMagar Candida (CHROMagar Microbiology); (ii) micromorphology on the rice agar; and (iii) by assimilation and fermentation tests including the ID 32C kit (bioMérieux). The used microorganisms were cultivated in a Mueller Hinton broth and agar plate (Difco, France).

B. Synthesis of Silver Nanoparticles and Their Characterization

The dispersion of silver NPs with a concentration of 5g/L of Ag was prepared by reduction of silver nitrate solution by sodium borohydride in a presence of sodium salt of polyacrylic acid. 7.87g of silver nitrate was dissolved in 865ml of distilled water followed by addition of 10ml of ammonia solution and 25ml of 45% sodium salt polyacrylic acid solution. After that 100ml of $NaBH_4$ solution (333.3mg in 100ml of distilled water) was added to the mixture of silver nitrate, ammonia and sodium salt polyacrylic acid under vigorous stirring. Immediately after addition of borohydride the dispersion turned dark-brown color indicating formation of colloidal silver. Such prepared dispersion of silver NPs was used for *in vitro* antimicrobial assay as well as for NanoSilver/methylcellulose hydrogel preparation without further modification.

The prepared silver NPs were characterized by set of instrumental techniques including dynamic light scattering (ZetaSizer NanoZS, Malvern, UK), transmission electron microscopy (Jeol 2010, Japan) and UV/Vis spectroscopy (Specord S600, Analytik Jena, Germany).

C. Preparation of NanoSilver/Methylcellulose Hydrogel

The methylcellulose based hydrogel was prepared by dispersing of 4g of methylcellulose in 60ml of boiling distilled water followed by intensive stirring for 30min. Subsequently, dispersion of methylcellulose in boiling water was refilled by 36ml of cold distilled water and cooled down to 50°C. This step has led to formation of hydrogel phase. After cooling down to 50°C silver NPs were incorporated into the methylcellulose hydrogel under vigorous stirring. The required amount of concentrated dispersion of silver NPs was added stepwise into the liquid methylcellulose hydrogel in order to achieve final silver concentrations equal to 25, 50, 100, 200 and 400mg/L. Mixture of silver NPs and methylcellulose hydrogel were kept stirring for 30 minutes at 50°C in order to obtain homogenous dispersion of silver NPs in methylcellulose hydrogel. The prepared NanoSilver/methylcellulose hydrogel was afterwards cooled down to laboratory temperature and used for *in vitro* antimicrobial assays.

D. In vitro Antimicrobial Assays

Antibacterial and antifungal activities of silver NPs alone were tested by using of standard microdilution method which enables to determine minimum inhibitory concentration (MIC) of an antibacterial substance. The testing was carried out on microtitration plates employing a method when we tested a dispersion of silver NPs 2-to-128 times, in the geometrical progression, diluted by addition of 100μL of Mueller-Hinton cultivation medium inoculated by tested bacteria and yeast strain at a concentration of 10^5 CFU•ml^{-1}. MIC value, expressing minimum concentration of tested compound that inhibited the growth of tested bacteria and yeasts, was determined after 24 hours of incubation at 3°C.

Antibacterial and antifungal activities of

NanoSilver/methylcellulose hydrogel were tested on agar nutrient. The prepared agar nutrient was transferred to sterilized Petri dish in laminar air flow box. After solidification of the media, bacteria or yeast culture was streaked on the solid surface of the media. In to this inoculated Petri dish, six small sockets with diameter of 7 mm were cut and filled up with 100μL of hydrogel of appropriate silver concentration as demonstrate schematic picture (Fig. 1). After 24h of cultivation at 37°C inhibitory zones formed around the hydrogel samples indicating microbial action were measured. For comparison antimicrobial action of pure methylcellulose hydrogel was also determined.

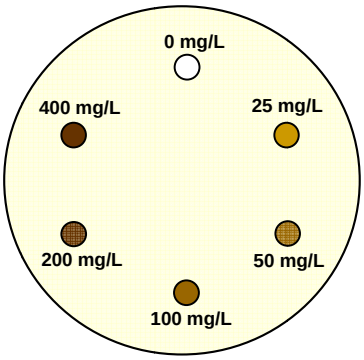

Fig. 1 Schematic image illustrating antimicrobial testing of NanoSilver/methylcellulose hydrogel on agar nutrient in Petri dish

III. Results and Discussion

A. Synthesis and Characterization of Silver Nanoparticles

Silver nanoparticles were synthesized by robust and effective method consisting in a reduction of silver nitrate via sodium borohydride. Development and optimization of the synthetic procedure leading to preparation of highly concentrated dispersion (5g/L of Ag) was necessary to ensure small volumes of silver dispersion being added in to the methylcellulose hydrogel in order to prepare NanoSilver/methylcellulose hydrogels with high amount of silver. On the other hand, such highly concentrated dispersion was unstable without subsequent stabilization, silver NPs had quickly formed large aggregates accompanied by rapid sedimentation. Thus sodium salt of polyacrilic acid of short polymer chain was used as a polymer stabilizing agent for the prepared silver NPs. Using this modifier the synthesized silver NPs were stable even at the concentration of 5g/L for several weeks. Such ordinary high stability of silver NPs was ensured due to the formation of adsorption layer of polyacrylate molecules on the surface of silver NPs. This adsorption layer of polyacrylate molecules allows steric stabilization and also electrostatic stabilization due to the negative charge on carboxyl groups of the polyacrylate molecules which are deprotonated in slightly alkaline media carrying on by presence of ammonium hydroxide.

The size of the prepared silver NPs was primarily measured by DLS technique and the resulting diameter of 10nm was directly confirmed using transmission electron microscopy (Fig. 2 (a)). From electron microscopy image can be seen that dispersion consist of small cubic silver NPs with diameter of 10nm. The absorption spectrum was measured immediately after preparation (see Fig. 2 (b)). In the UV–vis absorption spectra of the prepared silver NPs, the narrow surface plasmon absorption peak located at the wavelength of 396nm confirms the nanodimensions of the prepared silver NPs.

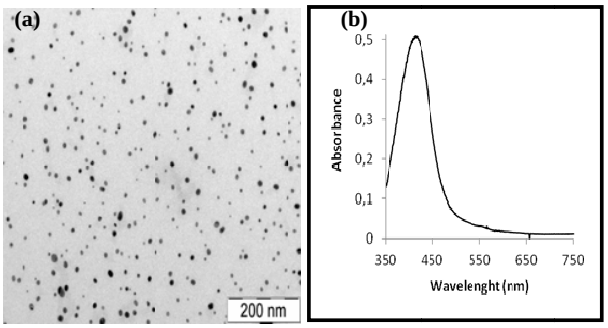

Fig. 2 (a) TEM image and (b) UV–vis absorption spectrum of silver NPs prepared via the reduction of silver ions by borohydride in the presence of sodium polyacrylate

B. Preparation and Antimicrobial Activity of NanoSilver/Methylcellulose Hydrogel

In the treatment of burn wounds, good hydration is considered as one of the most important factor that facilitates rapid wound healing and epithelia recovery. Usage of various natural and synthetic hydrogels developed for burn wound healing is often limited because of their poor mechanical properties and low rates of fluid absorption and retention. In this study, we used methylcellulose hydrogel which is considered as suitable hydrogel having desired properties such as hydration and high fluid retention ensuring optimal conditions for wound healing. Another important role facilitating burn wound healing is to prevent microbial colonization of wounds. This property can be achieved when the hydrogel is enriched by antibacterial substance. Classical antibiotics are limited by bacterial resistance which finally complicates treatment of burns. Therefore the main objective of this study was to prepare suitable hydrogel with good antimicrobial properties without any concerns connected with bacterial resistance.

These above mentioned requirements meet the NanoSilver/methylcellulose hydrogel and together with simplicity of its low cost preparation could be appropriate formulation in a treatment of burns and wounds. Silver NPs alone showed high antimicrobial activity as proved their MICs obtained from standard microdilution method. MIC of silver NPs varied from 0.84mg/L to 3.38mg/L depending on the used bacteria and from 0.21 to 0.42mg/L depending on the tested *Candida* sp. (Table I). It must be emphasized that such high antimicrobial activity is only given by silver NPs because control samples containing solutions of sodium borohydride and sodium salt of polyacrylic acid at concentrations corresponding to those of in dispersion showed no antibacterial activity (Table I).

TABLE I
MINIMUM INHIBITORY CONCENTRATIONS (MG/L) OF SILVER NPS IN THE FORM OF DISPERSION AND CONTROL SAMPLES CONTAINING NABH4 AND SODIUM POLYACRYLATE SOLUTIONS

	Minimum inhibition concentrations (mg/L)		
Bacteria/Yeast	Silver NPs	$NaBH_4$	Sodium polyacrylate
Escherichia coli 3954	1.69	-	-
Klebsiella pneumoniae 2486 (ESBL)	3.38	-	-
Pseudomonas aeruginosa CCM 3955	0.84	-	-
Pseudomonas aeruginosa 532	0.84	-	-
Staphylococcus aureus 3953	1.84	-	-
Staphylococcus aureus 4591 (MRSA)	1.84	-	-
Staphylococcus epidermidis 879	0.84	-	-
Staphylococcus epidermidis 901	0.84	-	-
Candida albicans I	0.21		
Candida albicans II	0.21	-	-
Candida tropicalis	0.42	-	-
Candida parapsilosis	0.42	-	-

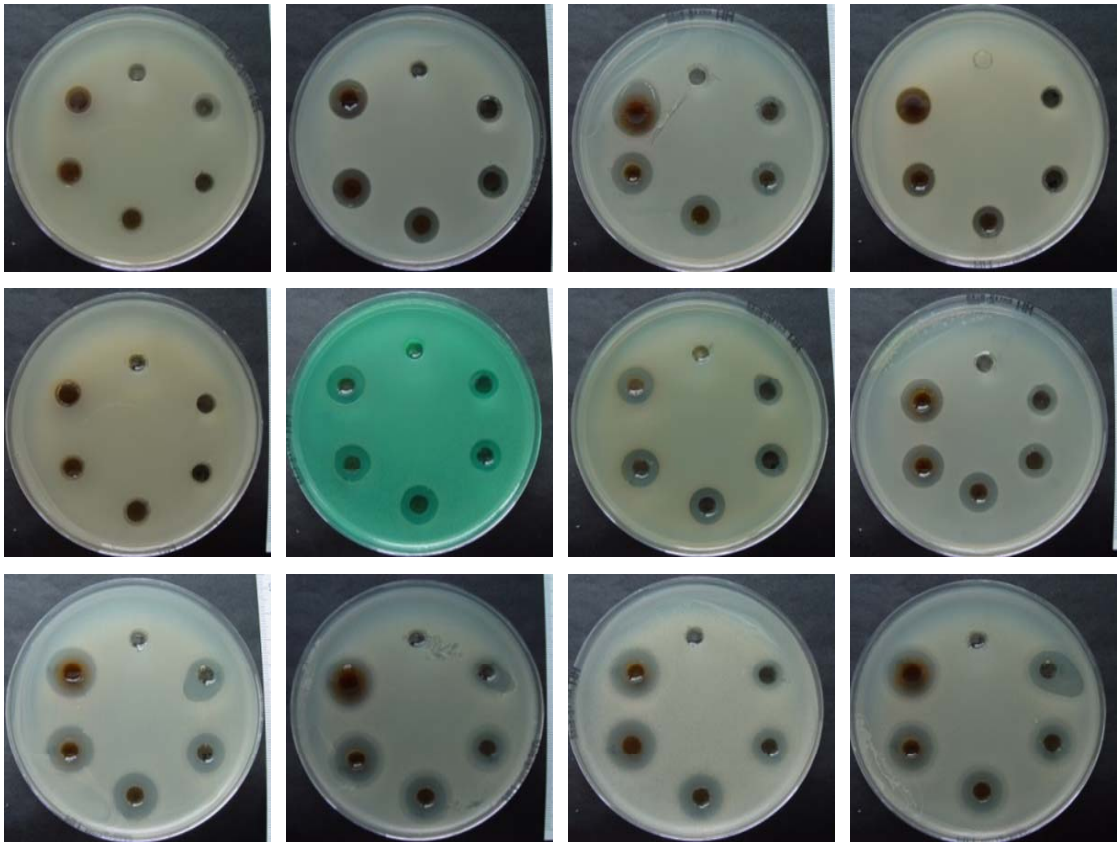

Fig. 3 Antibacterial activity testing of NanoSilver/methylcellulose hydrogel at various silver concentrations on agar nutrient against (a) *Escherichia coli* 3954, (b) *Staphylococcus aureus* 3953, (c) *Staphylococcus epidermidis* 879, (d) *Staphylococcus aureus* 4591 (MRSA), (e) *Klebsiella pneumoniae* 2486 (ESBL), (f) *Pseudomonas aeruginosa* CCM 3955, (g) *Pseudomonas aeruginosa* 532, (h) *Staphylococcus epidermidis* 901, (i) *Candida albicans* I, (j) *Candida albicans* II, (k) *Candida tropicalis* and (l) *Candida parapsilosis*

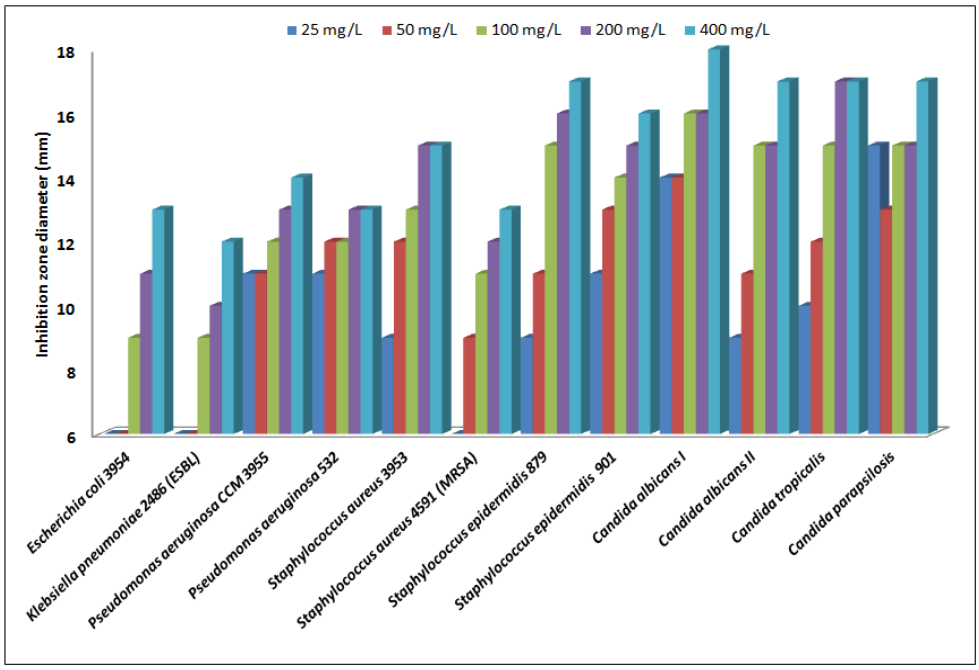

Fig. 4 Inhibition zone diameters of NanoSilver/methylcellulose hydrogel at various silver concentration against tested microorganisms

Such high antimicrobial activity of silver NPs themselves was responsible for high antimicrobial activity of prepared NanoSilver/methylcellulose hydrogel. The antimicrobial efficiency of the NanoSilver/methylcellulose hydrogel was tested using nutrient agar media. Fig. 3 shows the typical antibacterial and antifungal test results of hydrogel where clear zone of bacterial inhibition within and round the sample after 24h incubation of the agar plate at 37 °C can be seen. Fig. 3 also shows that no antimicrobial inhibition zone is formed in the case of pure methylcellulose hydrogel without silver NPs. Antimicrobial activity of NanoSilver/methylcellulose hydrogel is clearly seen from Fig. 3 following visible inhibition zones around hydrogel. Diameters of inhibition zones varied with type of tested microorganism and silver concentration used in the gel from 9 to 18mm (Fig. 4). Inhibition zones and therefore antimicrobial activity increased with increasing concentration of silver in NanoSilver/methylcellulose hydrogel which can be seen from Fig. 4. Antimicrobial efficiency of NanoSilver/methylcellulose hydrogel was higher against yeasts compared to bacteria and the reason why is that yeast are more susceptible to silver NPs as it was also observed from MIC values obtained in microdilution test. NanoSilver/methylcellulose hydrogel showed the best antibacterial performance against *Staphylococcus epidermidis* 901 and *Staphylococcus epidermidis* 879 where diameter of inhibition zones up to 17mm at the highest used silver concentration were observed. Antibacterial activity of NanoSilver/methylcellulose hydrogel to other tested bacteria was similar. Lowest antibacterial activity of NanoSilver/methylcellulose hydrogel was observed against *Escherichia coli* 3954 and *Klebsiella pneumoniae* 2486 (ESBL), the lowest silver concentration 25mg/l used in test showed no antibacterial effect.

Recent studies demonstrate that nanoparticles embedded into nano/micro/hydrogels or in situ generation of nanoparticles inside the gel networks leads to novel and advanced materials that can be applied directly to various biomedical applications [21]–[25]. On the other hand the preparation of some hydrogels is rather complicated. In our work the main criteria for selection of methylcelluose hydrogel are its biocompatibility, optimal properties such as hydration and high fluid retention, low cost and easy manipulation for the preparation of resulting NanoSilver/methylcellulose hydrogel.

IV. Conclusion

We have demonstrated a facile and low cost way to produce NanoSilver/methylcellulose hydrogel that can be directly used for various antimicrobial applications. Silver NPs used for the NanoSilver/methylcellulose hydrogel preparation were synthesized using rapid and robust reduction method enabling to prepare highly concentrated and stable dispersion of silver NPs with diameter of 10nm. NanoSilver/methylcellulose hydrogel showed high antimicrobial activities against broad spectrum of pathogenic microorganisms and therefore can be considered as a promising topical antimicrobial formulation for treatment of burns and wounds.

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