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Original article

Bioengineered mannan sulphate capped silver nanoparticles for accelerated and targeted wound healing: Physicochemical and biological investigations

Megha Mugade[a], Milind Patole[b], Varsha Pokharkar[a,*]

[a] Department of Pharmaceutics, Bharati Vidyapeeth University, Poona College of Pharmacy, Erandwane, Pune 411038, Maharashtra, India
[b] National Centre for Cell Science, NCCS Complex, Pune University Campus, Ganeshkhind, Pune 411007, Maharashtra, India

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ABSTRACT

In the present study, surface functionalized mannan sulphate silver nanoparticles (MS-AgNPs) were prepared and assessed for their wound healing potential. 20 nm sized, spherical MS-AgNPs were prepared by one pot synthesis approach wherein the sulphated polysaccharide mannan sulphate (MS) played dual role of reducing as well as capping agent. The crystalline MS-AgNPs exhibited surface plasmon resonance centered at 400 nm along with −32.40 mV zeta potential. These stable MS-AgNPs showed enhanced cytocompatibility, targeting potential and cellular uptake in murine macrophages, human skin fibroblasts and human keratinocytes as compared to citrate reduced silver nanoparticles (C-AgNPs). In the *in vivo* excision and incision wound models, MS-AgNPs as hydrogel formulations indicated better efficacy than the conventional and marketed silver formulations. Thus, the synthesized MS-AgNPs depicted a promising potential for site-specific topical delivery in accelerated wound therapy.

1. Introduction

Wound healing is a multi stage dynamic process which mainly involves the activation and recruitment of various types of cells, such as keratinocytes, macrophages and fibroblasts [1]. These cells express a large amount of mannose receptors, which make them amenable to receive ligand molecules effectively [2,3]. Therefore, they have expeditiously turned into an attractive receptor-specific target for both diagnosis as well as treatment of diseases [4–6]. Previous reports demonstrated that sulphated polysaccharide can strongly recognize mannose receptor [7,8]. Thus, it was thought that receptor specific cell targeting through surface functionalized nanoparticles may hold a promising potential [9]. *In vivo* excision and incision models are more reproducible and well-established animal wound models indicative of greater preclinical importance in the development of new therapeutic entities for wound healing [10,11].

The past decade has witnessed silver nanoparticles (AgNPs) as one of the most explored nanocarriers for diverse biomedical applications, biodiagnostics, biosensors, anti-bacterial as well as biocatalyst uses [12–14]. Customarily silver has long been renowned for their anti-bacterial efficacies which have been further enhanced with auxiliary activities at the nanoscale. Therefore, current research has been focused on the development of AgNPs like distinct inorganic nanomaterials. As of now, the preparation and stabilization of AgNPs through conventional physico-chemical approaches suffers from the shortcomings of high energy consumption, extreme physical conditions, involvement of toxic solvents and use of harsh chemicals along with the risk of colloidal nanoparticulate solutions being contaminated with various redundant byproducts. As silver nitrate and silver sulphadiazine like silver compounds get neutralized by anions like chloride and bicarbonate in body fluids causing argyria upon prolonged use, these topical preparations detain the healing process [15–17]. Thus, cytotoxicity and cosmetic abnormalities limit their wound healing potential. Due to these limitations, there is a need to design biocompatible and efficacious AgNPs in order to meet the therapeutic wound healing requirement.

Now-a-days, biomimetic methods based on the microorganisms and plant extracts are being endeavored owing to the ease of synthesis, ecofriendly benign nature, reduced toxicity and enhanced stability of nanoparticles. Various reports have revealed the use of microorganisms, fungi and plant extracts for green synthesis of AgNPs [18–25]. However, involvement of these natural resources fails to illustrate the exact molecule involved in the

* Corresponding author.
E-mail addresses: vbpokharkar@yahoo.co.in, varsha.pokharkar@bharatividyapeeth.edu (V. Pokharkar).

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reduction based synthesis. Additionally, the slower rate of biosynthesis hampers the use of such biological mediators. To overcome these shortcomings, the approach has been shifted toward exploring biomacromolecules and remained limited to gellan gum, chitosan, pullulan, dextran, porphyran and hydroxyl ethyl cellulose [26–32]. Beyond all studied and reported biomacromolecules, mannan sulphate (MS) remained unexplored for synthesis of AgNPs till date. In the present work, a novel approach for synthesis of AgNPs using MS has been explored. MS polysaccharide possessing the backbone of D-mannose units is obtained through mannan, which is isolated commercially from the cell wall of baker's yeast (*Saccharomyces cerevisiae*) [33]. MS has been found as a potent inhibitor of human immunodeficiency virus type 1 and many other enveloped viruses [34]. The aim of this study was to explore the role of MS as a reducing and capping agent in the synthesis of AgNPs. Further, it was envisaged that the MS capped AgNPs (MS-AgNPs) would exhibit enhanced uptake across the cells expressing the mannose receptor through their targeting potential. In our previous study, *in silico* molecular docking concluded that MS polysaccharide exhibited higher binding with cysteine-rich domain (Cys-MR) of the mannose multilectin receptor [35]. These interesting structural properties with targeting potential to the mannose receptors serve as valuable lead for the synthesis of nanoparticles.

The sulphate functionality attached to a primary hydroxy group present in MS polysaccharides serves as reducing as well as capping agents during synthesis of AgNPs [31]. Mannan sulphate was synthesized and characterized as per the method cited in our earlier report [36]. Thus, we propose that MS will play multiple roles during the synthesis of AgNPs for site-specific delivery to the skin cells, including the macrophages, keratinocytes and fibroblasts.

In the work, MS, a sulphated macromolecule was explored as reducing as well as a stabilizing agent in the synthesis of AgNPs. MS capped AgNPs were further characterized for physicochemical parameters through UV-Visible spectroscopy (UV), transmission electron microscopy (TEM), zeta potential, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) measurements and stability evaluation. The synthesized MS-AgNPs were further screened for *in vitro* cytotoxicity assay, cellular uptake studies, *ex vivo* diffusion study, *in vivo* excision and incision wound healing activity. These synthesized MS-AgNPs loaded hydrogel were compared with control, blank hydrogel, citrate reduced AgNPs (C-AgNPs) and marketed silver sulphadiazine cream for assessment of therapeutic wound healing potential.

2. Materials and methods

2.1. Materials

Silver nitrate ($AgNO_3$), mannan from *S. cerevisiae* (MW 43,000 Da), yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) and Texas red were procured from Sigma Aldrich, USA. Sodium citrate, ultra-pure nitric acid and hydrogen peroxide were obtained from Fluka Chemical Corp, USA. Carbopol® 980 NF (poly acrylic acid polymer) was gifted from Noveon, India. All other chemicals and reagents used in the experiments were of analytical grade. All the samples were prepared in the Milli Q water system (Nanopure Diamond by Barnstead, USA).

2.2. Methods

2.2.1. Synthesis of silver nanoparticles

Mannan sulphate (MS) was prepared as per the method reported in our earlier publication and characterized using well established methods [36]. Aqueous silver nitrate ($AgNO_3$, 1×10^{-3} M) solution was reduced to AgNPs by heating at 50 °C for 10 min in 0.01% w/v MS solution at basic pH 11 to yield a yellowish brown color dispersion of MS-AgNPs. The concentration of MS was varied (0.005, 0.01, 0.1, 0.2, 0.5 and 1% w/v) to estimate its effect on synthesis of MS-AgNPs. Subsequently, MS-AgNPs dispersion was purified by centrifugation (Allegra 64 R, Beckman Coulter, USA) at 10,000 RPM for 30 min and redispersed in Milli Q water.

Citrate reduced AgNPs (C-AgNPs) were synthesized *via* the well established Turkevich method wherein sodium citrate was involved in the reduction of silver nitrate [37,38].

2.2.2. Preparation of AgNPs loaded hydrogels

Topical gels were prepared by the cold mechanical method with some modifications [39,40]. The required quantity of Carbopol® 980 NF (1% w/v) was weighed and sprinkled slowly on the surface of AgNPs dispersion and continuously agitated by mechanical stirrer for 0.5 h. Triethanolamine was added in order to neutralize the gel up to pH 5.5. This AgNPs loaded hydrogel formulation was characterized labeled and packaged under sterile conditions at room temperature (25 °C) for further use.

2.2.3. Characterization of silver nanoparticles

2.2.3.1. UV-Visible spectroscopy measurements. The surface plasmon resonance (SPR) of MS-AgNPs dispersion was monitored by UV-Visible spectroscopy (Jasco V-470 Dual Beam spectrophotometer model, Japan).

2.2.3.2. Transmission electron microscopy (TEM) measurement. For transmission electron microscopy (TEM) measurement, the sample was prepared by drop casting of MS-AgNPs dispersion on the carbon coated copper grid. Measurements were performed on an instrument operated at an accelerated voltage of 300 kV with a lattice resolution of 0.14 nm and point image resolution of 0.20 nm. Particle size distribution analysis was carried out using ImageJ software (USA).

2.2.3.3. Zeta potential measurement. Surface charge on MSAgNPs was determined by zeta potential measurements using the zeta potential analyzer (Brookhaven Instruments Corporation, NY).

2.2.3.4. X-ray diffraction (XRD) measurements. For XRD measurements, the film was prepared by casting MS-AgNPs dispersion on glass substrates by simple solvent evaporation technique at room temperature. Diffraction measurements were performed at a current of 30 mA, scan rate of 0.38° min^{-1}. Average particle size (D) was calculated using Debye–Scherrer formula

$$D = \frac{0.89 \cdot \lambda}{W \cdot \cos\theta}$$

where D is the particle size; W is the full width at half-maximum of the 111 peak in radians; λ is the wavelength of the X-ray source and θ is the diffraction angle.

2.2.3.5. Fourier transform infrared spectroscopy (FTIR) measurements. FTIR measurements of MS and MS-AgNPs were recorded using FTIR-4600 spectrophotometer (JASCO, Japan) in the diffuse reflectance mode of 400–4000 cm^{-1}.

2.2.4. pH, electrolyte and storage stability study

The stability of MS-AgNPs was evaluated under varied pH and electrolytic conditions and over long term storage time. In the pH stability evaluation, the pH of AgNPs dispersion was varied between pH 2 and 12 using 0.1 M hydrochloric acid and 0.1 M