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Synthesis and characterization of honey based hybrid dental implants with enhanced antibacterial activity

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Abstract: Dental implants are commonly used for tooth replacement tools due to their good oral rehabilitation and reconstruction capacities. Dental implants treatment for natural teeth is desired to achieve successful implants treatment with improved osseointegration through promotion of mammalian cell activity and prevention of bacterial activity. Honey is potentially known for its antimicrobial and antibacterial potential, specifically for burns and wound healing. In this study, honey based silver nanoparticles were synthesized using various concentrations of honey. The synthesized HNY-AgNPs, MSN and HNY-AgMSN were characterized for their surface Plasmon resonance using UV spectroscopy, Hydrodynamic diameter using Zetasizer. Morphology using AFM. Furthermore, surface functional groups were characterized using FTIR spectroscopy at 4cm^{-1} resolutions. The developed hybrid nanoparticles were tested for their anti-bacterial activity at concentration of 3000µg/mL. It was found HNY-AgNPs was active against both bacterial strains i.e, *Streptococcus mutans* and *streptococcus aureus*. HNY-AgNPs-MSN hybrid implant demonstrated potential new type of dental implants, which can offer an effective design for the fabrication of advanced dental implants.

Keywords: Honey, dental implants, mesoporous silica nanoparticles, synthesis, characterization, anti-bacterial activity.

INTRODUCTION

With the advancement in dental implantology, several techniques are used to increase the success of dental implants treatment. The most common and popular material used for dental implants is metallic material due to their high corrosion resistance, stable mechanical property, and biocompatibility (Smeet *et al*., 2016; Park *et al*., 2017; Branemark 1977; Liu *et al.,* 2017). Regardless of these benefits of metallic material, the partial osseo integration between the surfaces implant and transplanted site become a serious problem after the treatment (Heo *et al*., 2016). Diverse research focused on this problem revealed that an ideal period for osseo integration is three to six months after the operation. The patient must undergo masticatory trouble during the osseo integration period. Besides, the bone and the soft tissues are more prone to periodontitis during this osseointegration period which causes the stimulation of inflammation and also causes bone loss. This required antibiotic treatment and eventually results in implants failure (Persson *et al.*, 2001).

Hence, it is a challenge to develop such a material that not only promotes osseo integration effectively but also suppress the bacterial biofilm formation. Focus on this, the research study propose a coherent and rational design honey based silver nanoparticles with mesoporous silica nanoparticles (HNY-AgNPs-MSN) that will provide the surface property for stimulating the osseo integration and enhanced wettability (Persson *et al*., 2001). Honey is commonly used for antimicrobial and antibacterial activity as well as used for infected wound healing. Due to this action of honey and easy availability and accessibility we aim to use honey to improve the surface property of metallic material.

Titanium implants are commonly used for dental practice and procedures and these implants could prodigiously affect the mechanical stability of implant as well as clinical outcomes. The implants surface properties produce direct impact on tissue reaction by affecting the protein adsorption and cell proliferation. Advancement and improvement in the surface modification methods have greatly increased the biological performance of titanium implants (Gittens *et al*., 2011; Landolt 2007).

For more than 50 years titanium implants have been used for their smooth surface texture. With the passage of time different modifications in the chemical and topographical alteration and variation of titanium has changed its surface topography. Many *in vitro* and *in vivo* studies on surface modification of titanium implants suggested that it promotes more prompt bone formation. The two main factors due to which implants surface are more prone to the infection are biofilm formation and compromised ability at tissue/implant interface (Albrektsson and Wennerberg 2004). We further determine and offer a material as dental implants that will control the bacterial cell and this material use as dental implants are cost effective.

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MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis of honey-based silver nanoparticles (HNY-AgNPs)

Natural honey acquired from the local market and silver nitrate ($AgNO_3$) were used in accordance with previously published protocol (Philips 2010). Briefly, (20-40 % v/v) solution of honey was prepared in DI water. Then freshly prepared $AgNO_3$ (1mM) was added to 15mL honey solution in different volumetric ratios and stir gently for 5 min. To trigger the reduction of silver ions the pH was adjusted to 8.5 by using sodium hydroxide (NaOH). Reduction can be characterized by changes formation of brownish color indicating the formation of honey silver nanoparticles (HNY-AgNPs).

Synthesis of mesoporous silica nanoparticles (MSN)

Mesoporous silica nanoparticles were prepared according to previously reported protocol (Vazquez *et al*., 2017). Briefly, Sol was prepared by mixing known volumes of ethanol/ water with ammonium hydroxide (NH_4OH), then CTAB was added, and the mixture was maintained under constant stirring for 20 min. Then, TEOS was added and under vigorous stirring for 30 min at room temperature. Once the reaction started the solution turns opaque. The white powder precipitated was filtered and washed with DI water. The particles were dried under ambient conditions. The obtained powder was then washed with methanolic HCl to remove surfactant and dried at room temperature. The hydrodynamic diameter using Zetasizer (Zetasizer Nano ZS90 Malvern Instruments, Malvern, U.K.). Morphology using AFM (AFM, Agilent 5500). Furthermore, surface functional group were characterized using FTIR spectroscopy IR-470 spectrometer (Shimadzu, Kyoto, Japan) at 4 cm^{-1} resolution.

Synthesis of HNY-AgMSN hybrids

Mesoporous silica nanoparticles comprise of hydrophobic cores which are actively used to deliver drugs at the diseased site. Passive coating technique was used to prepare HNY-AgMSN and proceeded by mixing HNY-AgNPs with different amounts of MSN followed by incubated for 24 h to facilitate the particles uptake within cores of mesoporous silica nanoparticles.

Characterization

The synthesized HNY-AgNPs, MSN and HNY-AgMSN were characterized for their surface Plasmon resonance using UV spectroscopy (Shimadzu, 1800 series). Hydrodynamic diameter using Zetasizer (Zetasizer Nano ZS90 Malvern Instruments, Malvern, U.K.). Morphology using AFM (AFM, Agilent 5500). Furthermore, surface functional group were characterized using FTIR spectroscopy IR-470 spectrometer (Shimadzu, Kyoto, Japan) at 4 cm^{-1} resolutions.

Anti-bacterial activity

Micro-Plate Alamar Blue Assay (MABA)

Stock solution 60mg/mL of all compounds were prepared in 100% DMSO and serially double fold diluted in Mueller Hinton Broth (MHB) in 96 well plate ranging concentration from (3000, 1500, 750, 375, 180, 90 and 45µg/mL) such that their final volume becomes 100µL (compound and media). Fully grown *Staphylococcus aureus* (ATCC 25923) and *Streptococcus mutans* (NTCT 10449) were 1000 times diluted in MHB media and 100 µL of this diluted bacterial media were added to all previous wells such that final volume (200µL) would contain bacterial load approximately (0.5-1.0 x 106 CFU/mL). Positive control contains only media and bacteria. Each concentration was run in triplicates. Plates were sealed and incubated at 37oC for 18-22h. Next day, all wells were visually checked to confirm the growth of bacteria. In all wells 20µL of 0.02% Alamarblue dye (Alfa Aesar) was added and incubated in a shaking incubator at 80 rpm and 37^{o}C for 2h. The change in color of Alamar dye from blue to pink indicated the growth of bacteria strains. For quantitative analysis, absorbance was recorded at 570nm and 600nm in Multiskan™ GO spectrophotometer (Thermo Scientific, USA). Percentage inhibition was calculated as mentioned by the protocol of Shaymaa *et al*., 2002.

STATISTICAL ANALYSIS

All the values are expressed as Mean ± SEM. The data were analyzed by one way ANOVA followed by LSD multiple comparison tests. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Surface plasmon resonance of developed HNY-AgNPs

To assure the synthesis of desired HNY-AgNPs UV-Visible spectroscopy was conducted, which was widely used for metallic nanoparticles characterization. The surface plasmon resonance of developed HNY-AG-NPs was depicted in fig. 1. Furthermore, the peak in SPR region revealed the slight polydisperse nanoparticles.

Preparation of mesoporous silica nanoparticles (MSN)

Mesoporous silica nanoparticles are recognized as potential absorbents due to its porous framework and its ability to tailor diverse targeting receptors and drugs (Akbar *et al*., 2022). In current study MSN was used as an absorbent with the aim to functionalize the prepared HNY-AgNPs onto its surface. The porosity of our MSN enables the entrapment of non-chelated organic molecules which thereby enhances the antibacterial efficacy. In our study mesoporous silica nanoparticles were prepared by Stober procedure which involves the hydrolysis of silyl ethers under basic condition in presence of surfactant such as CTAB and after development of white precipitate the surfactant was removed with the aid of acidic methanol enables the porosity within silica nanoparticles.

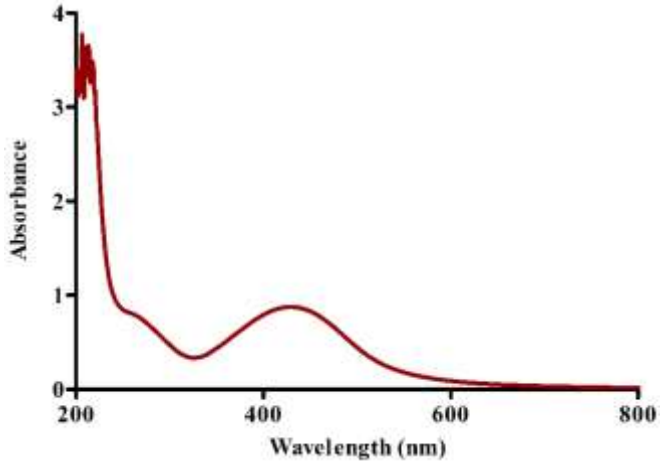

Fig. 1: Surface plasmon resonance peak of designed HNY-AgNPs.

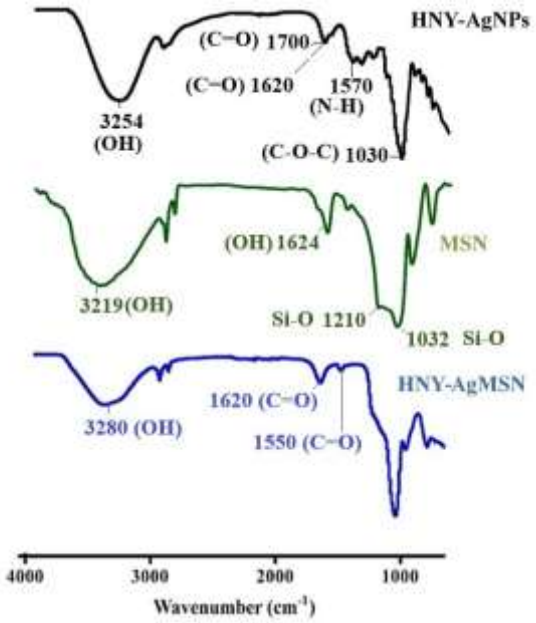

Fig. 2: FTIR spectra of HNY-AgNPs, MSN and HNY- AgMSN

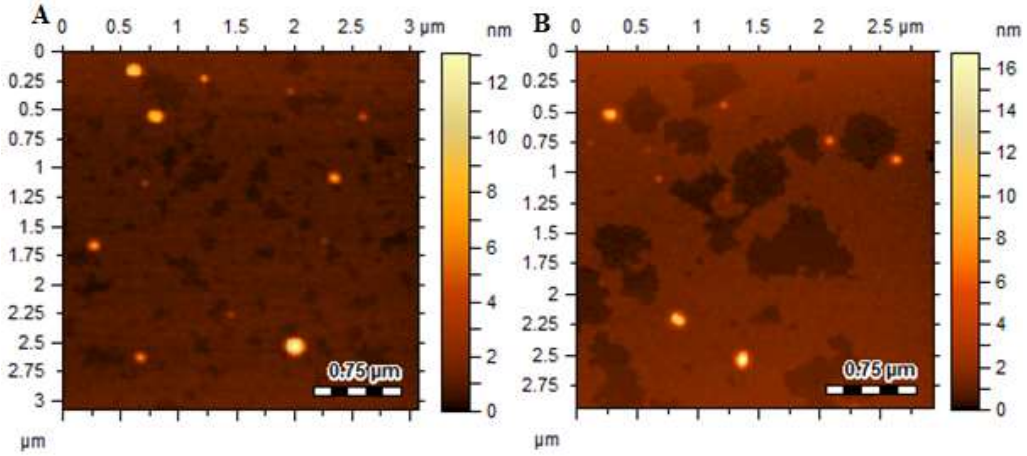

Fig. 3: AFM images of HNY-AgNPs and HNY-AgMSN

Table 1: Average Size, zeta potential and polydispersity index of developed nanoparticles.

Samples	Average Size (nm)	Zeta Potential (mV)	PDI
HNY-AgNPs	144 ±1.5	-5.63 ±0.32	0.319 ±0.021
MSN	107 ±7.4	-2.53 ±0.96	0.293 ±0.12
HNY-AgMSN	154.6 ±15.3	-6.42 ±1.74	0.23 ±0.042

Table 2: Minimum inhibitory concentration (MIC) of compounds against Staphylococcus aureus and Streptococcus mutans.

Sample	Staphylococcus Aureus	Streptococcus Mutans
H-AgNPs	160-320µg/mL	80-160µg/mL

FTIR spectroscopic analysis of developed nanostructures

FTIR spectroscopy was used to analyze the possible biomolecules responsible coating and stabilization of silver nanoparticles using honey. The FTIR spectra of HNY-AgNPs show two prominent peak of amide I and II of protein at 1620cm^{-1} and 1570cm^{-1} (fig. 2) corresponds to C=O stretching and N-H bending vibrations. The band at 1375cm^{-1} was also observed corresponds to C-O stretching mode of carbonyl and glyosidic moieties present in honey. An intense band at 1030cm^{-1} corresponds to C-O-C stretching of sugars in honey. Furthermore, the absence of C=O at 1700cm^{-1} of carboxylic moiety and presence of asymmetric stretching vibration of amine group at 3286 cm^{-1} shows that the stabilization onto the surface of proteins form honey onto the surface of AgNPs were facilitated via carboxylate ions. The FTIR spectra of MSN (fig. 2) show characteristic absorptions at 3219cm^{-1}, 1624cm^{-1}, 1210 cm^{-1}, and 1032cm^{-1} corresponding to OH (stretching and bending) and Si-O groups (Akbar *et al*., 2022). The FTIR spectra of HNY-AgMSN show prominent peak of amide I and II at 1620 and 1550cm^{-1} form protein along with peak increased NH_2 stretching vibration peak at 3280cm^{-1} indicates that HNY-AgNPs was successfully incorporated with silica nanospheres.

Average size, zeta potential, polydispersity index and morphology

The average size of HNY-AgNPs, MSN and HNY-AgMSN was presented in table 1. The increment in size occurred in the case of HNY-AgMSN which may be due to the incorporation of HNY-AgNPs within cavities of MSN. The study on colloidal behavior of NPs revealed that PDI value was greater than 0.5, indicates size broadening of NPs (Stewart *et al*., 2000). The PDI values of HNY-AgNPs, MSN and HNY-AgMSN were shown in Table 1. Morphological analysis via AFM revealed that the developed nanoparticles are nearly spherical in morphology (fig. 3) indicating higher stability.

Anti-bacterial activity

In the present study, two different gram-positive bacterial strains (*S. aureus* and *S. mutans*) were used to assess the antibacterial activity. The developed hybrid nanoparticles were tested at highest concentration of 3000µg/mL. HNY-AgNPs was found to be active against both bacterial strains. MIC of HNY-AgNPs against *S. aureus* (ATCC 25293) and *S. mutans* (NTCT 10449) were found to be 160-320µg/mL and 80-160µg/mL respectively (Table 2).

DISCUSSION

Surface treatment is commonly used now a days to develop advanced dental implants for improving quality. In this study we used material-based surface treatment which is honey. Honey is known for its antibacterial properties, and its good biocompatibility provides a tool to improve the function of biomaterial. The change of the surface properties can control the function of both bacterial cells and offer new design for dental implants.

Metallic nanoparticles by nature inherited with optical absorption characteristics in the UV-Visible region termed as surface plasmon resonance (SPR). The production of HNY-AgNPs is indicated by the potent absorption peak at 413 nm which is in correlation with previously published reports (Philip, 2010). To optimize desired HNY-AgNPs, the concentration of honey varied form 20-40 % and the concentration of silver nitrate was held constant (1mM) and the UV-Visible characterization revealed that the suitable ratio for further analysis was found to be 20% honey (Kumar *et al*., 2020). Furthermore, Honey is a complex mixture of compounds possess diverse skeletal and pharmacological characteristics. The increment in concentration of honey leads to the increased chelation of compounds onto the surface of AgNPs and this phenomena lead to aggregation of AgNPs that is the reason increment in honey concentration greater than 20 % leads to SPR broadening. The pH plays a significant role in the preparation of AgNPs, since honey is a pool of various compounds that are acidic in nature such as carboxylic acid, phenols and several others. When the pH of the medium increases to 8.5, this facilitates the production of gluconic acid from glucose and which increases the chelation onto the surface of AgNPs (Hosny *et al*., 2017).

Honey contains various essentials compounds including glucose, fructose, vitamins, proteins etc. (White 1978).

Proteins are considered as macromolecules comprised of carboxylic, amine and amide moieties and when undergoing nano-stabilization with silver nanoparticles carboxylate ion and amine groups are responsible for effective chelation of with silver ions and the presence of these group was confirmed by FTIR analysis of HNY-AgNPs. HNY-AgNPs was coated onto the surface of MSN which is evident from FTIR spectra and due to abundant hydrobhobic porosity of MSN the unreacted nanoparticles got trapped in it and which also contributes to the enhancement of overall antibacterial efficacy.

Polydispersity index (PDI) value is the measure of colloidal homogeneity of nanostructures. The PDI values of less than 0.5 in our study suggesting that our nanoparticles showed more uniform dispersion, indicating higher colloidal stability of our developed nanoparticles (Kawish *et al*., 2020). Zeta potential corresponds to the difference in potential between dispersed phase and dispersed medium. Increment in zeta potential values leads to decrease in aggregation and which leads to higher colloidal stability. In our study the greater intensity of negative zeta potential values may indicate higher physical stability of the developed nanoparticles. Previous studies revealed that NPs having size below 1000 nm can easily permeate biological barriers to transport drugs at the desired site of action in increased amounts (Kong *et al*., 2019). AFM images revealed that our developed nanoparticles are nearly spherical in morphology which contributes in overall colloidal stability of nanoparticles (Couteau & Roebben, 2011).

Many bacteria are present in the oral cavity which rapidly forms biofilm. Most commonly *Streptococcus mutans* which is a gram-positive bacterium stimulates early biofilm formation. The developed hybrid nanoparticles were tested at highest concentration of 3000g/mL. HNY-AgNPs was found to be active against *Streptococcus mutans* and *streptococcus aureus*. Furthermore, cell proliferation and biofilm formation assay are in progress which is required for to get information about the cell attachment and proliferation on the surface of dental implant. Nanoparticles may interact with the walls of bacterial cells, affecting the permeability of the cell membrane and disrupting the activity of respiratory chain enzymes (Khorrami, Jafari Najafabadi, Zarepour & Zarrabi, 2019). Increased amounts of free radicals develop during this case, affecting the functioning of different cellular organelles. Nanoparticles may penetrate bacterial cells by rupturing the cell wall and interacting alongside the sulfur and phosphorus components of enzymes and DNA, resulting in the death of the bacterium. Furthermore, the hazardous consequences associated with the release of silver ions are also related to these nanoparticles. The nanoparticles release silver ions that effectively penetrate bacteria, causing damage (Kawish *et al*., 2020; Lahiri *et al*., 2020). Attributing the antibacterial properties of silver nanoparticles to just one mechanism is not feasible. Each of these variables may affect bacterial deactivation. However, depending on the physical and chemical properties of nanomaterials and the physiological characteristics of bacteria, one of these pathways dominates. The proposed method includes nanoparticles sticking to the cell wall, which then alters membrane permeability and disables internal cell components. The nanoparticles have reduced activity against Gram-positive strains due to their strong peptidoglycan cell wall, which hinders nanoparticle penetration, unlike the weaker cell wall in Gram-negative bacteria (Danish *et al*., 2022).

CONCLUSION

No material or metallic alloy can be considered biocompatible and this does not eliminate or omit titanium. The biomaterial used for dental implants can alter the oral environment to an erratic degree and that may cause allergic reactions within the oral tissues (Mosges, 2002). Therefore, we demonstrate that Honey based hybrid has good alternative to the traditional surface treatment. HNY-AgNPs-MSN hybrid implants can offer an effective design for the fabrication of advanced dental implants. The outcome of antibacterial activity of the HNY-AgNPs offers a coherent design for the development of other implants with the changed surface properties.

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