Research Article

Azadirachta indica (Neem) Gum Coated Gold Nanoparticles as Nano-go-karts to Dispatch Haloperidol Across Blood-Brain-Barrier.

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ABSTRACT

Here efforts to develop a nano sized delivery systems that can cross the blood brain barrier to deliver anti-schizophrenic drug Haloperidol, is reported. Chemically synthesized gold nano particles of 20-22 nm diameter, coated with peptides of *Azadirachta indica* (Neem) Gum were used as carrier of Haloperidol. Neem gum coated gold nano particles were highly stable and did not need any linker for attachment of the drug. The drug loading efficiency was found to be 92%. So far as drug release kinetics is concerned initially for first 3 hrs there was a burst of drug release and after 24 hrs sustained release of Haloperidol for a period of 168 hrs was noted. The drug release profile of Haloperidol under physiological conditions followed Higuchi model, suggesting that the drug release is through diffusion. Moreover, Haloperidol showed unidirectional diffusion through the matrix of NG which is used to coat Gold Nano Particles.

Keywords: Blood-Brain-Barrier, Haloperidol, anti-schizophrenic drug, Gold Nanoparticles, Neem Gum.

INTRODUCTION

Blood Brain Barrier (BBB) organized with the capillary endothelial cells obstructs penetration of therapeutic agents like antibody, peptides, proteins etc.[1,2] The brain saviour also hinders the passage of small molecules like drugs. Consequently, drug delivery to brain for treatment of neurodegenerative diseases is challenging and very less amount of drug acts on the brain cells.[3,4] Increase in the drug concentration to achieve the desired effect subsequently leads to side effects.[3,5] To overcome this, it has been reported earlier that permeability coefficient (P_{app}) of nanoparticles with size 30 nm and below is higher; also amino-Q-dots were found to be getting more actively transported through *in vitro* BBB model than other surface charged Q-dots.[6]

Gold Nano-Particles (GNPs) have been extensively used by the scientists as drug delivery system owing to their less cytotoxicity, facile synthesis, solubility and ease of functionalization.[7,8] High surface area to volume ratio of GNPs facilitates their diffusion and also improves interaction of these nanoparticles with surrounding environment.[9] GNPs offer efficient release of the drug to the target site, controlled release as well as minimum usage of drug. Up till now, GNPs such as nano-triangles[10,11], Nano-rods[12,13], Nano-Cubes[14], Nano-shells[15,16] etc. have been used as carrier vehicle for drug delivery. Apart from these, because of absorbance in the near Infra Red (IR) region, GNPs have proven to be noteworthy in the photothermal therapy of tumor.[17,18]

GNPs can be synthesized by chemical as well as biological method[7-9], wherein, chemically synthesized GNPs require a linker like GSH, Cysteamine HCL etc. to be attached so that drug can be covalently attached onto them. Moreover, use of chemical linker can create toxicity and stability issues and hence is generally considered unfavourable. Till date GNPs have been synthesized with the assistance of algae[19,20], microbes[21,22], fungi[23,24] and plant systems[7,10] are found to be thermodynamically more stable and less toxic.

These GNPs get naturally coated with peptides, which can be used as linkers to attach therapeutic agents and hence are more biocompatible.[7,10] The size of GNPs synthesized by biological method is not easy to control resulting in more polydispersity, which is perhaps the only drawback. However, with the help of Sucrose Density Gradient Centrifugation (SDGC) our group has successfully separated GNPs size-wise.[25]

In the present paper we report synthesis of *Azadirachta indica* gum (neem gum) coated GNPs for the delivery of Haloperidol (Halo), an anti-psychotic drug. Halo is butyrophenone, which is used for the treatment of schizophrenia, a neurodegenerative disorder, characterized by the increased dopamine receptors on the cell membrane.

The study involved synthesis of GNPs by previously reported chemical method[26]; furthermore, GNPs were coated with Neem Gum (NG), a secondary metabolite obtained from the *Azadirachta indica* tree. NG polymer coating provides the anchoring sites for drug attachment.

Moreover, NG stabilizes GNPs acting as capping agent and prevents their agglomeration. Stabilized GNPs were further conjugated to Halo and its drug release kinetics was studied. This is first attempt to integrate chemical

and biological method with intent of adapting their advantages for further medical applications.

MATERIALS AND METHODS

Materials

Aurochloric acid ($HAuCl_4$), Tri-sodium citrate and Halo were purchased from Sigma–Aldrich, USA. Neem gum (NG) was obtained by piercing the bark of the locally grown *Azadirachta indica* tree. Experiments were carried out using nanopure water. In order to remove the traces of metal contaminants glasswares were washed with Aqua Regia.

Preparation of NG stock

Stock of 3000 ppm NG was prepared by dissolving 60 mg of NG in 20 mL nanopure water and then filtered through 0.22 µ filter to remove the dust particles. It was stored at 4˚C until further use.

Synthesis of GNPs

A stock solution of 50,000 ppm $HAuCl_4$was prepared in nanopure water. In order to use 100 ppm $HAuCl_4$, 0.06 mL stock solution was added in 30 mL boiling solution of reaction vessel containing 1.5 mL Tri-sodium citrate (pH 10). The mixture was boiled till the appearance of wine red color.

Characterization

UV-Vis spectroscopic (Lambda 25 PerkinElmer, USA) measurements were carried out using nanopure water as a reference. Clean quartz cuvette having path length 1 cm was used to record the spectra.

Morphology of GNPs was studied using High Resolution Transmission Electron Microscopy (HR-TEM) on a Carl Zeiss Microimaging, GmbH, Germany. 2-3 drops of colloidal solution of GNPs was loaded onto the copper grid of with dropper.

Involvement of diverse functional groups and molecular interactions as well as molecular orientation of the complexes was verified using Fourier Transformed Infra Red Spectroscopy (FTIR) on a MAGNA-550, Nicolet instruments, USA. The sample was prepared by loading 0.1 mL of GNPs in aqueous form onto the source.

Coating of NG on GNPs

For coating of NG on to GNPs, 3.33 mL from NG stock (3000 ppm) was added to mixture of GNP colloidal solution (24 mL) and nanopure water (12.66 mL); resulting into a total volume of 40 ml and kept at room temperature for 24 hours. Coating of NG onto GNPs (NG-GNPs) were initially characterized by UV-Visible spectrophotometer and later on confirmed by FTIR.

Stability testing of GNPs and NG coated GNPs using flocculation parameter

The stability of GNPs and NG-GNPs was checked by analyzing the changes in the optical properties in response to the varying concentrations of NaCl. Salt solution was added to cuvette containing GNP and NG-GNP solution and UV-Vis spectrum was recorded. Procedure was repeated for both GNP and NG-GNP solution with increasing concentrations from 0.017 to 3.4 M of NaCl to observe whether there is any shift in peak; as compared to the original peak of GNP and NG-GNP respectively. Flocculation Parameter (FP) is an empirical term used for measurement of integrated absorbance between longer wavelengths (400-600 nm in case of GNPs)[27]. The equation used to calculate the integrated absorbance is as follows:

$$P = \int_{400}^{600} I_{Abs}(\lambda)dx$$

Where, P – Flocculation Parameter, I_{Abs} – Intensity of absorbance and λ – wavelength

Attachment of Haloperidol onto NG-GNP

1000 ppm stock solution was prepared by dissolving 10 mg Halo in 10 mL absolute ethanol. In order to use 200 ppm Halo, 4 mL of stock solution was added in 8 mL of NG-GNPs and was stirred at 200 rpm for 4 hours.

Attachment of Halo to NG-GNP was analyzed spectrophotometrically and by FTIR spectroscopy.

The resultant Halo-NG-GNP conjugate was dialyzed overnight against nano-pure water to remove unbound Halo. Unbound drug concentration was calculated using standard calibration curve of Halo (straight line equation y=0.002x).

Drug loading efficiency (DLE) of NG-GNPs was calculated using following equation:

$$DLE = \frac{\text{Theoretical amount of drug loaded} - \text{Free drug}}{\text{Theoretical amount of drug loaded}} \times 100$$

In vitro release of Halo from Halo-NG-GNP

Dialyzed Halo-NG-GNP solution was taken in pre-activated dialysis bags (3 mL) and transferred to beaker containing 70 mL of phosphate buffer solution of pH 7.2. The drug release study was conducted at 37°C with stirring at 150 rpm. To measure the drug release content, samples (3 mL) were periodically removed and replaced back in the buffer solution.

The amount of released Halo was analyzed with a spectrophotometer at 267 nm and calculated using standard calibration curve of Halo (straight line equation y = 0.002x). The experiments were performed in triplicate.

With precise control of the Halo-NG-GNP complex, the release of the drug can be tuned to achieve a desired kinetic profile.

Four of the most common kinetic profiles i.e. Zero order, First order, Higuchi and Hixson-Crowell model were considered.

RESULTS AND DISCUSSION

Synthesis of GNP

GNPs synthesized using $HAuCl_4$ and 1% tri-sodium citrate (pH 10), the latter acting as reducing agent as well as stabilizing agent, showed colour transformation from pale yellow to wine red after heating and a sharp peak at 519 nm (Fig. 1), which is due to the Surface Plasmon Resonance (SPR).

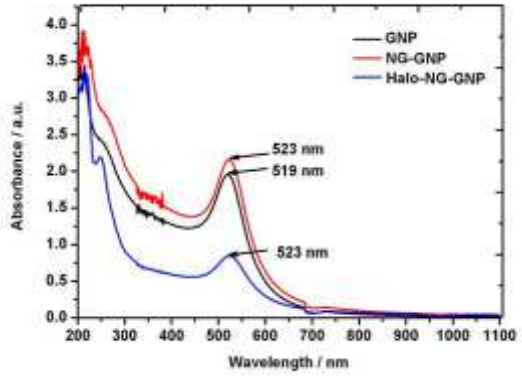

Figure 1: UV-visible spectra of the GNP, NG-GNP and Halo-NG-GNP

Confinement of electrons on surface of GNPs gives rise to unique optical property and sharp peak infers the presence of mono-dispersed GNPs. After coating of NG, a minor red shift in SPR peak from 519 nm to 523 nm was observed (Fig. 1); this may be because of increase in size of GNPs due to NG coating. There was no peak shift observed after attachment of Halo, however, intensity of peak decreased which may be indicative of interaction between proteins on NG coated nanoparticles surface and Halo. These interactions were further confirmed by FTIR studies.

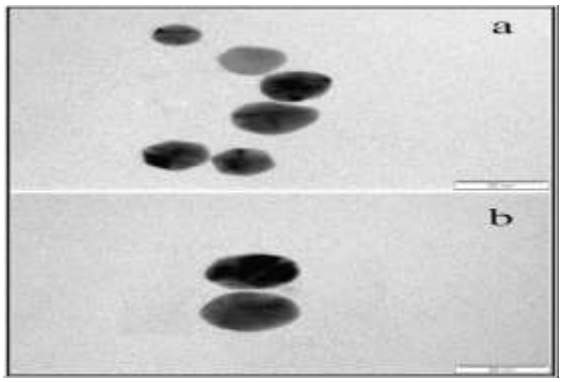

Figure 2: TEM image of the GNPs – (a) GNPs before capping with NG and (b) Increase in size of GNPs after capping with NG

TEM image (Fig. 2a) shows 16-18 nm sized chemogenic GNPs whereas figure Fig. 2b displays biologically capped GNPs with size around 20-22 nm confirming the coating of NG onto the bare GNP surface which according to the previous studies these can cross the BBB [6] and hence can serve as potential drug delivery vehicles.

FTIR analysis

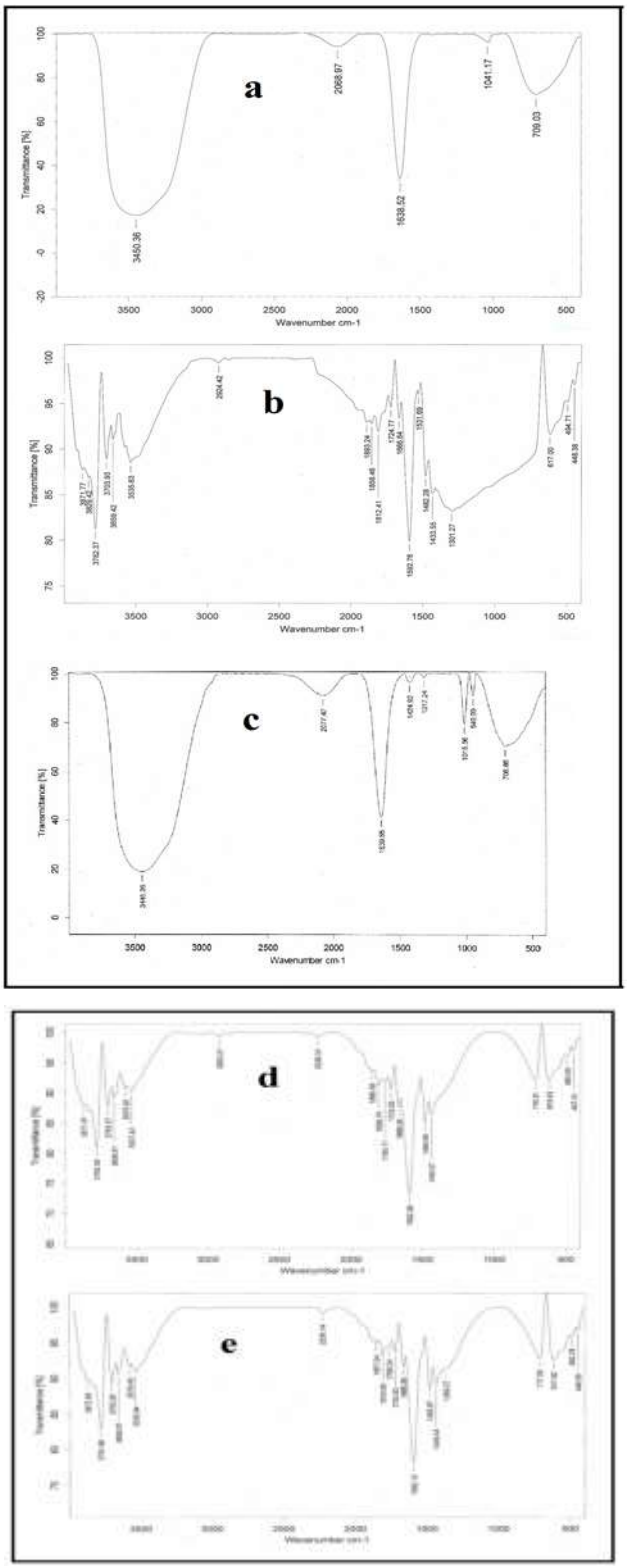

Figure 3: FTIR spectra of (a) *Azadirachta indica* (Neem) gum, (b) Bare GNPs, (c) Neem gum coated GNPs, (d) Haloperidol and (e) Final complex (Halo-NG-GNP)

FTIR Spectra of Neem Gum (NG) - displayed in Fig. 3a shows a broad peak at 709.03 cm^{-1}, which corresponds to $–CH_2$ bend of alkanes. A minor peak at 1041.17 cm^{-1} depicts C-O bonding of alcohol. An intense and narrow peak at 1638 cm^{-1} represents –NH bend and C=O stretching of amide linkages; this is expected to be due to the proteinaceous content of the neem gum i.e. amino acids present in gum like alanine, aspargine, glycine etc. and sugars present viz. fructose, galactose etc. respectively.[28] A broad and weak peak at 2068 cm^{-1} corresponds to the –N=C=S iso-thyocyanates. A broad and intense peak at 3450.36 cm^{-1} was obtained due to –OH stretching, that is mainly due to the water molecules present in the aqueous extract used for analysis.

FTIR Spectra of NG-GNP- In the Fig. 3c, FTIR spectra of NG coated GNPs, displays 4 new peaks arising at 949.99, 1015.56, 1317.24 and 1424.92 cm^{-1} that were not present in Fig. 3a and 3b. These peaks are attributed to S-O stretch of sulfonates; C-O-C stretching vibrations may be due to C=O bond between NG and GNP; C-O stretch of carboxylic acid as well as bending of –OH respectively. Slight increase in transmittance of peak at1639.65 cm^{-1} (Fig. 3c and 3a) indicates possible interaction between NG and GNPs. Moreover, there was decrease in transmittance (90%) as well as shift of peak at 2077.47 cm^{-1} (Fig. 3c) which was originally at 2068.97 cm^{-1} (Fig. 3a) of 94% of transmittance corresponding to–N=C=S isothiocyanates displaying possibility of NG coating.

FTIR Spectra of bare Halo and Halo-NG-GNP Conjugates - Peak at 716.31cm^{-1} present in the FTIR spectra of Halo (Fig. 3d) is due to the bending of CH_2, which is also present in FTIR spectra (Fig. 3e) of Halo-NG-GNP complex (717.05 cm^{-1}) with slight shift and found to be absent in NG-GNP spectra indicating interaction between NG-GNP and Halo. Peak at 2923.21 cm^{-1} in Halo spectra is due to the -CH stretch which disappeared in Halo-NG-GNP complex possibly due to the formation of new bonds in the complex.

Stability studies of GNPs using Flocculation parameter

The effect of increasing concentrations of NaCl on the optical properties of GNPs is shown in Fig. 4a. Since the GNPs were coated with NG extract, proteins and peptides present on the surface of GNPs resisted the agglomeration in the presence of NaCl even up to the concentration of 2.84 M. In contrast, bare GNPs (Fig. 4b) were not able to withstand the higher concentrations of NaCl and agglomeration occurred at 0.085 M NaCl concentration.

The initial peak of NG-GNPs was at 523 nm which red shifted to 526 nm after addition of salt solution. Moreover, after addition of any further amount of NaCl solution, the peak was found to be stable with decreasing intensity as a consequence of increasing volume.

Red shift indicates that there may be presence of dipole interaction between adjacent nanoparticles induced by change in dielectrics of the solution on addition of NaCl.

Folding of capping proteins will also determine the fate of NG-GNP, wherein, no aggregation will occur if hydrophilic groups are exposed and exposure of hydrophobic group will lead to agglomeration.

Here no aggregation of NG-GNP complex occurred demonstrating their outstanding stability.

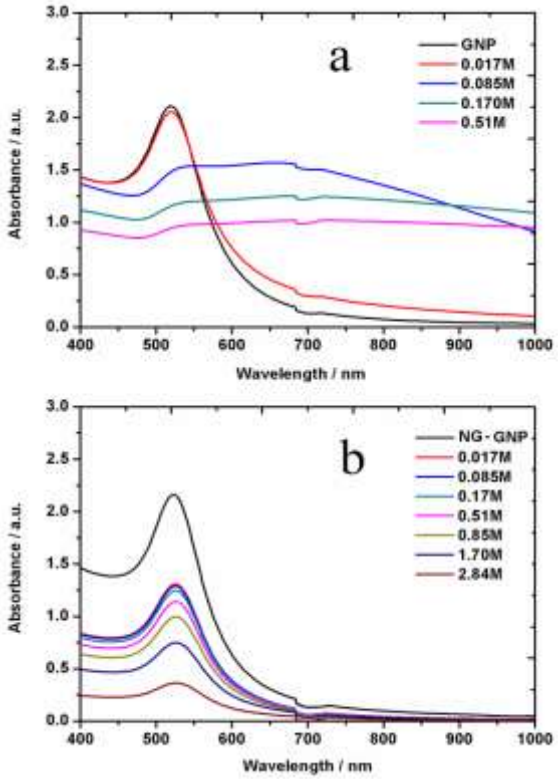

Figure 4: Effect of the increasing salt concentration on (a) Bare GNPs and (b) NG-GNPs, showing the increased salt tolerability of GNPs after NG coating

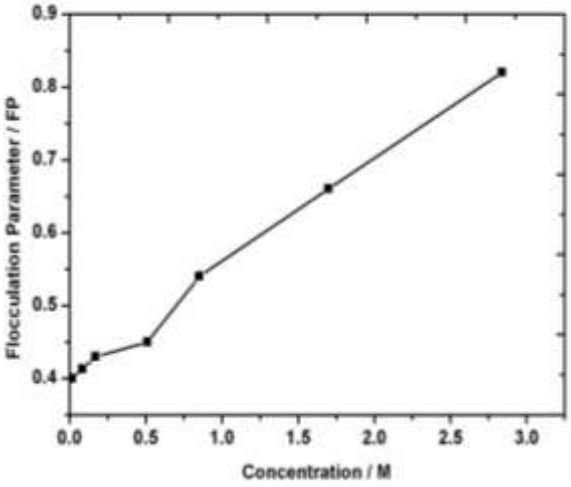

Figure 5: Flocculation parameter of NG-GNPs showing their exceptional stability.

Fig. 5 shows that FP is positively correlated with concentration of salt. Capping proteins being only determinants of FP in this case, it can be inferred that at particular pH, competence of capping protein to resist agglomeration is directly proportional to FP. This stability

derived from capping proteins makes GNP ideal candidates for drug delivery; also capping proteins provide sites for the attachment of drug instead of synthetic linkers thus reducing toxicity.

Drug release kinetics

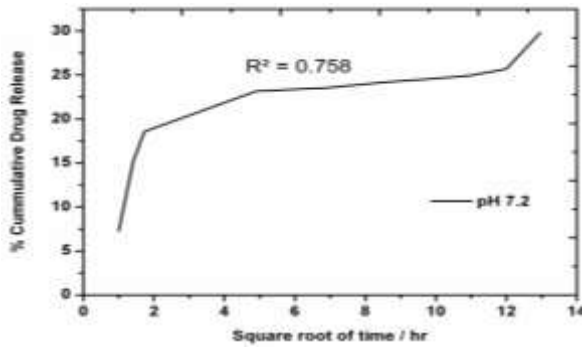

Figure 6: % Cumulative release of Haloperidol with respect to square root of time in *In-vitro* conditions.

The loading efficiency of Halo was found to be 92.53%. Fig. 6 shows drug release pattern of Halo with respect to time at physiological conditions (37°C, pH 7.2). Drug release after 1st, 2nd and 3rd hour was 7.3%, 15.23% and 18.58% respectively indicating initial burst of Halo. After 24 hours sustained release (23.18%) of Halo was observed up to 168 hours, has been observed by several other researchers also.[29] Researchers have studied polymeric nanoparticles and end group effects on release of Halo. Here we assessed impact of natural polymer coated GNP on drug release based on large surface area provided by GNP and matrix offered by the natural polymer covering around GNPs resulting into sustained Halo release. The rationale of the drug release pattern was estimated using different statistical models. These models assess the type of drug release profile under physiological conditions. Among four most extensively used models (Zero Order, First Order, Higuchi and Hixon-Crowell), Halo is following Higuchi model based on highest correlation value (R^2) obtained. Higuchi model assumes the direct relation between cumulative drug release and square root of time. The model explains basically a drug release is through diffusion. In our case, Halo showed unidirectional diffusion through the matrix of NG which is used to coat GNPs.

CONCLUSION

Chemically synthesized GNPs enveloped with coating of Neem Gum were found to be more stable than bear GNPs and hence can serve as more efficient drug delivery vehicles than pure GNPs.

The size of biologically capped NG-GNPs synthesized was found to be between 20-22 nm which is ideal and relating to previous studies by Hanada[6], permeability coefficient of these GNPs will be higher thus allowing them to pass through BBB. Another added advantage is NG-GNP has outstanding drug loading efficiency as a consequence of natural linkers present due to coating of NG on chemical GNP. Overall NG-GNP is ideal carrier for Halo.

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