International Journal of Pharmaceutics 471 (2014) 439–448

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical nanotechnology

Structure function attributes of gold nanoparticle vaccine association: Effect of particle size and association temperature

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ARTICLE INFO

Article history:
Received 12 April 2014
Received in revised form 2 June 2014
Accepted 2 June 2014
Available online 5 June 2014

Keywords:
Gold nanoparticle
Nanoparticle protein interaction
Vaccine formulation
Association thermodynamics
Structure function studies

ABSTRACT

Many biotherapeutic applications of gold nanoparticles make use of conjugated or adsorbed protein moieties. Physical parameters of association such as particle size, morphology, surface chemistry and temperature influences the protein-nanoparticle association and thereby their interaction with the biological environment. In present study, effect of size of chitosan reduced gold nanoparticles (CsAuNPs) and association temperature on structure and function of tetanus toxoid (TT) vaccine has been investigated. CsAuNPs were synthesized in the sizes of 20 ± 3, 40 ± 5 and 80 ± 7 nm followed by loading of TT. Binding process of CsAuNPs with TT was investigated at their predetermined micro molar concentrations. Upon binding of TT onto CsAuNPs, particle surface was characterized using X-ray photoelectron spectroscopy. CD spectroscopic evaluation of TT bound 20 nm CsAuNPs led to 75% reduction in secondary structure of TT and thereby compromised immune function. Binding of TT with 40 and 80 nm sized CsAuNPs did not cause significant modifications in secondary structure or function of TT. Thermodynamic studies using temperature dependent fluorescence spectroscopy revealed an increase in association constants with the temperature. Based on thermodynamic data three phases in CsAuNPs and TT association process were traced. Samples from these distinct phases were also investigated for immunological recognition. *Ex-vivo* interaction of TT-CsAuNPs with TT positive and negative sera followed by relative change in particle size and zeta potential was studied. The findings here suggests prominent role of particle size and association temperature on adsorbed TT structure and function. Such studies may help in engineering functional nanotherapeutics.

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1. Introduction

Gold nanoparticles (AuNPs) are being pursued for delivery of therapeutic proteins due to ease of synthesis and surface modification (Ghosh et al., 2008). Therefore their interaction with therapeutic proteins is having enormous attention of the researchers worldwide. Gold nanoparticles are biocompatible in nature and are effectively up-taken by live cells (Alkilany and Murphy, 2010). Thus they are considered as a potential delivery system for vaccines. Gold nanoparticles are known to have broad spectrum of applications in cancer therapy and diagnosis (Jain et al., 2008; Cai et al., 2008; Llevot and Astruc, 2012). However their role in deciding structural and functional fate of protein can potentially be investigated with nanoparticle-protein binding studies. There are few reports suggesting influence of particle size (Pramanik et al., 2008), shape (Chithrani, 2011), charge (Lee et al., 2010), density and surface coating (Cho et al., 2011) onto the protein structure. However investigations into effect of such physical parameters onto both structure and function of protein need to be undertaken. Effect of nanoparticle shape on protein structure and function has been previously reported (Gagner et al., 2011). We report here, effect of particle size and association temperature on tetanus toxoid (TT) protein structure and function.

Based on biological and medical literature about nanoparticulate carriers, their biopharmaceutical applications as well as toxic responses upon exposure to proteins can potentially be studied (Silvia et al., 2010). We have previously demonstrated immunological synergy of chitosan reduced gold nanoparticles (CsAuNPs) with TT immunogen (Barhate et al., 2013). Obtained immunological synergy generated our considerable interest in understanding optimum process parameters governing fate of nanoparticle

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http://dx.doi.org/10.1016/j.ijpharm.2014.06.002
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protein association. We hypothesized particle size and temperature might have an influence onto the structural fate and functional properties of active proteins such as tetanus vaccine. Therefore in present study, high resolution transmission electron microscopy, dynamic light scattering and zeta potential measurements were carried to trace colloidal properties of CsAuNPs. Fluorescence and circular dichroism spectroscopy techniques were used to investigate effect of CsAuNPs on TT. These studies were carried with respect to CsAuNPs size and association temperature. X-ray photoelecteron spectroscopy was used to understand surface-chemistry of TT associated CsAuNPs. TT specific immunological assay was used to investigate effect of process variables onto immune recognition of TT. We further put emphasis on the enumeration of the protein-nanoparticle binding process and thereby effects of nanoparticle size and association temperature on structure and function of TT. Such studies may lead towards better understanding the effect of vaccine and nanoparticle association conditions on vaccine structure and thereby on the surrounding biological environment.

2. Materials and methods

2.1. Materials

Tetanus toxoid bulk (3250 Lf/ml) was procured from Serum Institute of India Ltd., Pune. Chitosan (degree of deacetylation 85%, molecular weight 45,000 Da) was a gift sample from A & E Connock Ltd., UK. Chitosan (degree of deacetylation 95%, molecular weight 45,000 Da) was purchased from Sigma (St. Louis, MO). Gold (III) chloride trihydrate 99.9% was purchased from Sisco Research Lab. (Mumbai, India). All glassware used in synthesis were prewashed with aqua-regia. DI water (Milli-Q) was used in all experiments. All other chemicals and reagents were of analytical grade.

2.2. Instrumentation

All transmission electron micrographs were obtained using FEI, Tecnai G2 20 ultra-TWIN instrument (Netherlands) operating at 200 kV, lattice resolution of 0.102 nm and point image resolution of 0.19 nm using TIA (Netherlands). Atomic absorption spectroscopic (AAS) measurements were made using Chemito Instruments AA-201. X-ray photoelectron spectroscopy (XPS) was performed on VG scientific ESCA-3000 spectrometer (UK) using non-monochromatized Mg Kα radiation (1253.6 eV). Absorption measurements were done on Jasco Dual Beam Spectrophotometer (Japan). Fluorescence emission measurements were made on PerkinElmer LS-50B spectrofluorimeter with thermostatically controlled sample holder (Massachusetts, USA) and UV-CD spectra were recorded on Jasco J-815 spectropolarimeter (Japan).

2.3. Surface functionalized nanoparticle synthesis

Three batches of chitosan functionalized AuNPs were prepared using our previously reported procedure (Barhate et al., 2013), with few modifications. Briefly, chitosan solutions (0.2, 0.3 and 0.4% w/v, respectively) were prepared using 1% acetic acid, to this aqueous chloroauric acid ($HAuCl_4$ 1.25×10^{-3} M) was added and resultant system was heated at 100 °C for 10 min. Obtained ruby-red colored dispersion was termed as batches A, B and C, respectively. Prepared batches of CsAuNPs were further used to study the particle-protein interaction.

2.4. Nanoparticle characterization

The Au concentration (in parts per million) was determined in final CsAuNPs samples using AAS. Wherein, standard calibration graph for Au solution was prepared. CsAuNPs samples were digested using aqua regia ($1HNO_3$:4HCl), diluted to known factor using DI water and processed using AAS. Transmission electron microscopy (TEM) samples were prepared by depositing 20 μl of sonic waves processed CsAuNPs sample on a carbon-coated copper grid and overnight drying under vacuum. Observations were made by placing the copper grid in airtight TEM assembly and recording images up to 125K× magnification. The monolayer of TT:CsAuNPs was prepared onto glass slide and subjected to analysis of surface chemistry using XPS technique.

2.5. Characterization of TT microenvironment

Intrinsic fluorescence of tryptophan in TT (95 μg/ml) was studied where, TT was excited at 295 nm and emission characteristics were recorded in the range of 300–400 nm. Parameters for excitation and emission were 10 mm path length cell and slit width of 7 nm. Interaction of TT with neutral (5 M acrylamide (Acr)), cationic (5 M cesium chloride (CsCl)) and anionic (5 M potassium iodide (KI)) quenchers was studied. The respective quenching data was analyzed using Stern–Volmer (Eq. (1)) and modified Stern–Volmer equation (Eq. (2)).

$$\frac{F_0}{F_c} = 1 + K_{sv}[Q] \tag{1}$$

$$\frac{F_0}{F_0 - F_c} = f_a^{-1} + (K_a f_a)^{-1}[Q]^{-1} \tag{2}$$

wherein F_0 and F_c are the relative fluorescence intensities, corrected using relative dilution factor, in absence and presence of quencher, respectively. K_{sv} is the Stern–Volmer constant for the respective quencher and $[Q]$ is resultant concentration of quencher. Term f_a refers to fraction of total accessible fluorescence to the quencher and K_a refers to resultant quenching constant for the accessible fraction. Stern–Volmer and modified Stern–Volmer plots for the native and denatured TT were studied to understand relative structural changes upon interaction with the charged quenchers. Deducing slop of Stern–Volmer plot gives K_{sv} values (Eq. (1)) and slope of modified Stern–Volmer plots yield $(K_a f_a)^{-1}$ further their ordinate gives value of $1/f_a$ (Eq. (2)).

2.6. Effect of temperature on TT-CsAuNPs association

Effect of temperature onto association process was carried out at fixed TT concentration (95 μg/ml) and 40 nm CsAuNPs (1 μM). The variable in this study was temperature (22 °C, 28 °C, 37 °C and 42 °C). We have quantified the fluorescence quenching by the relation where F_0 and F are fluorescence intensities in the absence and presence of CsAuNPs, respectively. It was assumed that there is an equilibrium in binding of proteins to NPs. Likewise, fluorescence quenching data was fitted into Eqs. (1)–(3) followed by calculation of binding constant K from plot of $\log[(F_0 - F_c)/F_c]$ verses $\log[Q]$. Briefly, calculation of the slope of plot yields value of n (number of binding sites) and intercept drawn on Y-axis gives value of $\log K$. Further calculating antilog of $\log K$ gives respective binding constant between nanoparticle and protein.

$$\log\left[\frac{F_0 - F_c}{F_c}\right] = \log K + n \log [Q] \tag{3}$$

Further, thermodynamic parameters of CsAuNPs and TT interaction were investigated. In an interaction wherein insignificant change in enthalpy (ΔH) occurs over the range of study temperature, thermodynamic parameters such as ΔH° and ΔS° or change in entropy can be determined using van't Hoff equation (Murphy, 1999) (Eq. (4))

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