CHAPTER 4

HYALURONIC ACID CO-FUNCTIONALIZED GOLD NANOPARTICLE COMPLEX FOR THE TARGETED DELIVERY OF METFORMIN IN THE TREATMENT OF LIVER CANCER (HepG2 cells)

4.1 INTRODUCTION

Primary liver cancer is the fifth most commonly diagnosed cancer and the third highest cause of death worldwide. Hepatocellular carcinoma (HCC) represents around 85% of all primary liver cancer (Bhalla et al. 2012). Even as the incidence of some cancers is declining, incidences of HCC is increasing around the world (El-Serag & Rudolph 2007, Parkin et al. 2005). AuNPs have become an important biomedical tool for scientists in cancer research because of the several advantages of AuNPs like high loading efficiency for the target substance, enhanced ability to cross various physiological barriers, and low systemic side effects (Patra et al. 2008; Patra et al. 2008; Patra et al. 2008; Dykman et al. 2012). Most of the AuNPs synthesized by chemical methods involve toxic chemicals that may affect the environment in addition to human health (Devi et al. 2015). In this era, a ‘green’ chemistry and bioprocess approach has attracted considerable attention as it helps to reduce the use of tedious techniques and harsh reducing agents. Our study focused on the use of cost effective, ecofriendly and biocompatible reducing agent for synthesis of AuNPs (Narayanan & Sakthivel 2008).
The eggplant (*Solanum melongena L.*) fruit contains ascorbic acid and phenolic acid; both of them are potent antioxidants (Matsubara et al. 2005). It seems that the eggplant extracts suppressed the development of blood vessels that are required for tumour growth and metastasis (Vinson et al. 1998). Carbohydrate-coated AuNPs have proved to be effective therapeutic substances improving the biocompatibility, targeting ability and producing higher efficiency in drug delivery (Rojo et al. 2004). Biocompatible naturally occurring polysaccharides such as chitosan (Bhumkar et al. 2007) and gellan gum (Dhar et al. 2008) were already reported as useful reducing agents for rapid synthesis of AuNPs and subsequently used for drug delivery applications. HA is a linear, anionic polysaccharide (Goddard & Gruber 1999).

HA was used as both the reducing and stabilizing agent in the formation of AuNPs in sizes ranging from 5 to 30 nm by thermal treatment, (Park et al. 2011). HAs have been widely used as novel drug carriers for target-specificity and long-acting delivery (Luo et al. 2002). They have been employed both as delivery vehicles and angiostatic agents in cancer therapy (Luo et al. 2000). As a drug carrier, a biocompatible linear polysaccharide of HA is highly efficient in targeted delivery to liver tissues with HA receptors such as the hyaluronan receptor for endocytosis (HARE) and the cluster determinant CD44 (Ahrens et al. 2001, Entwistle et al. 1996). MET HCl is a biguanide antihyperglycemic agent, used for the management of Type II diabetes (Wiernsperger & Bailey 1999, Marchesini et al. 2001).

Although it is known that diabetes increases the relative risk of HCC, a number of studies show that treatment of diabetics with MET is associated with a reduced risk of HCC (Donadon et al. 2010; Donadon et al. 2009). Some major problems associated with the drug are its low
bioavailability and short half-life period (Graham et al. 2011). To overcome these problems, we have developed a method to synthesize H-AuNPs functionalized with HA for targeted MET delivery. To check the toxicity of the prepared AuNPs, both in-vitro and in-vivo studies were used. In this study, we have used zebrafish embryo as a model to evaluate the toxicity of the AuNPs, because of its homology to the human genome and comparable human tissue types (BarIlan et al. 2009). In this paper, we describe an ecofriendly sunlight mediated method for the preparation of biocompatible AuNPs by reducing HAuCl₄ using eggplant fruit extract in the presence of HA. The HA-MET conjugate was successfully synthesized by the amide bond formation between amine groups of MET and carboxylic groups of HA activated with 1-ethyl-3-(3- dimethyl aminopropyl) carbodiimide and N-hydroxysulfosuccinimide.

4.2 EXPERIMENTAL

4.2.1 Material

Eggplant fruit extract was used in this study. Metformin was obtained from SRL Limited, Mumbai (India). Hydrochloroauric acid (HAuCl₄), Sodium hyaluronate, the sodium salt of Hyaluronic acid, with a molecular weight of 12 kDa, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N- hydroxysuccinimide sodium salt (NHS), and MTT (3- (4, 5-dimethylthiazolyl-2)-2, 5- diphenyltetrazolium bromide) were obtained from Sigma-Aldrich Chemicals (India). HepG2 cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, (India). Antibody to β-actin, Bcl-2, p53, caspases-3, cleaved caspase-3, caspase-9, cleaved caspase-9, PARP, cleaved PARP, and cyclinD1, cyclin E, cdk-4, cdk-6, cdk-2 were purchased from (Santa Cruz Biotechnology, CA,
USA) and Neo Markers USA. Millipore milliQ water was used for all the experiments. All other chemicals and reagents were of analytical grade.

4.2.2 Methods

4.2.2.1 Preparation of the extract

*Limonia acidissima* L fruits fruit was finely chopped into small pieces and boiled in 100 ml of sterile double distilled water for 3 min. The extract was centrifuged at 10,000 rpm for 10 min to remove any undesired impurities. This extract was filtered and stored at 4°C for further experiments.

4.2.3 Synthesis of MET-H-AuNPs

10 ml of 1mM HAuCl₄ solution was added to HA solution (1000 µg in 10 ml) and optimized eggplant extract (150 µl) mixture and it was irradiated under natural sunlight. It was observed that the yellow colored gold solution turned into wine red color within 20 min. The synthesized AuNPs were purified by centrifugation at 15000×g for 15 min followed by repeated washing to remove uncoated HA. (Satish et al. 2009). H-AuNPs (1500 µg as per ICP-OES calculation) was mixed with EDC (800 µg) and NHS (900µg) and the solution was incubated for 30 min to modify the carboxyl groups of HA with NHS. After that, MET (1000 µg) was added to the activated H-AuNPs, and was stirred overnight to complete the reaction. Finally, the resulting solution was poured into the prewashed dialysis membrane tube with a molecular weight cut-off of 3.5 kDa and dialyzed against a large excess amount of 100 mMNaCl aqueous solution, 25% ethanol and pure water. After lyophilization for 3 days, the obtained dark reddish brown color MET-H-AuNPs powder was stored at 4°C for future use.
4.2.4 Characterization of H-AuNPs and MET-H-AuNPs

Preliminary characterization of H-AuNPs was carried out by FTIR, HR-TEM and particle size and zeta potential analysis method.

4.2.4.1 Fourier Transform Infrared Spectroscopy (FTIR) measurements

The FTIR spectra of pure metformin and EPI-FA-AuNPs were recorded on a Perkin-Elmer Spectrum-One instrument in the diffuse reflectance mode at a resolution of 4 cm\(^{-1}\) in the range 400-4000 cm\(^{-1}\) on KBr pellets.

4.2.4.2 X-Ray Diffraction (XRD) spectrum of green synthesized gold nanoparticles

The crystalline nature of the synthesized AuNPs was studied by XRD (Bruker D8 series) analysis. The sample was prepared using a thin powder of AuNPs on a clean glass slide and the analysis was carried out using monochromatic Cu K\(\alpha\) radiation running at 40 kV and 30 mA with a step size of 0.005°

4.2.4.3 High Resolution-Transmission Electron Microscopy (HR-TEM) analysis

High resolution-transmission electron microscopic pictures were recorded in a JEOL-JEM-2100 HRTEM operated at 200 kV. For the HRTEM analysis a droplet of aqueous solution of synthesized AuNPs was spread onto a carbon coated copper grid (300 meshes) and dried under IR lamp. Micrographs were taken both in the transmission mode and in the diffraction mode.
4.2.4.4 Particle size and zeta potential measurements

The hydrodynamic particle size and the nanoparticle charge quantified as zeta potential, was determined on a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) instrument equipped with a He–Ne laser operating at 632.8 nm and a scattering detector at 173°. DLS and nanoparticle charge measurements, were determined using the same instrument at 25°C. The polydispersity index (PDI) was also quantified to determine the particle size distribution range.

4.2.5 In-Vitro Stability Studies

1 ml of H-AuNPs was incubated with 0.5 ml each of 0.9% NaCl saline and phosphate buffer saline (PBS) (pH 1.2, 4.5, 6.8 and 7.4) respectively at 37°C for 48 h, and were analyzed spectrophotometrically.

4.2.6 In-Vivo Toxicity Study of H-AuNPs in Zebra Fish Embryos

Fertilized eggs of zebra fish were obtained from the natural mating of adult zebra fish and embryos were collected within 2 h of spawning. Six healthy embryos from fertilized eggs, approximately 2 h post fertilization (hpf), were transferred to each well of a 24-well plate containing 1 ml of the E3 medium. The embryos were exposed to different concentrations of H-AuNPs for 4 days (Kim et al. 2013). Duplicates were maintained without the H-AuNPs as control. Toxicity was assessed by studying the hatching rate, percentage survival rate and morphology changes in the embryos.
4.2.7 Loading Efficiency

From the total concentration of MET used for loading onto H-AuNPs, some of it remains unattached. The unloaded drug was removed by separating the supernatant after centrifugation (Eppendorf centrifuge 5430R) at $15,000 \times g$ for 15 min. Free MET present in the supernatant was determined by UV-Vis spectrophotometrically by measuring at 237 nm. The percentage of drug loading efficiency was calculated using the following formula (4.1).

\[
\text{Drug loading efficiency} = \frac{\text{Theoretical amount of drug loaded} - \text{Free drug}}{\text{Theoretical amount of drug loaded}} \times 100
\]

(4.1)

4.2.8 In-Vitro Drug Release Characteristics

MET conjugated H-AuNPs (equivalent to 1000 µg of MET) was dialyzed against 100 ml of sodium phosphate buffer, (pH 5.7 and 7.4), at $37^\circ C$ with continuous stirring at 100 rpm. 1 ml of sample was withdrawn at specific time intervals and analyzed spectrophotometrically. Sink condition was maintained by replacing equal volume of buffer. The release studies were performed in triplicate and the average was taken. The percentage of drug release was calculated by using following formula (4.2).

\[
\text{Release} (\%) = \frac{\text{Released metformin}}{\text{Total metformin}} \times 100
\]

(4.2)

4.2.9 In-Vitro Cytotoxicity Studies

Cell viability was measured by MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphe- nyl tetrazolium bromide) assay described by Mossman et al.
Cell viability was measured by MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide) assay described by Mossman et al. (1983). Approximately (5 × 10^3 cells/well) cells was placed in each well of 96 well plates and incubated for 24 h for attachment. After incubation, supernatant media was replaced with an equal amount of fresh media containing different concentrations of EPI-FA-AuNPs. After incubation for 48 hrs, MTT solution was added to the plate at a final concentration of 0.5 mg/mL and incubated for 4 hr in dark at 37°C. The resulting formazan crystals were dissolved by DMSO. Cell Viability was calculated by measuring optical density at 570 nm in ELISA reader (Bio-Rad Instruments Inc., USA).

4.2.10 Apoptosis Study

The influence of MET-H-AuNPs in inducing apoptosis in the liver cancer cells was confirmed using staining methodology (Karthik, Sankar, Varunkumar & Ravi Kumar 2014). The influence of EPI-FA-AuNPs in inducing apoptosis in the liver cancer cells was confirmed using acridine orange (AO) and ethidium bromide (EB) [1 mg/ml for each AO and EB in PBS] staining methodology (Karthik, Sankar, Varunkumar & Ravi Kumar 2014). In brief, $5 \times 10^5$ cells/well were cultured on a cover slip in a 6-cell well plate and incubated all night long for attachment. After attachment, the cells were treated with fresh medium containing pure-EPI and EPI-FA-AuNPs (100µg/ml). After 36 h incubation, the cover slip was removed and stained with AO/EB (10µl) for thirty minutes and washed with PBS for removing the excess staining dye. Images were captured by Nikon Eclipse inverted fluorescence microscope.
4.2.11 DNA Fragmentation Assay, Flow Cytometric Analysis and Western Blotting

The DNA fragmentation assay was performed according to the method of Sambrook, Russell, 2001). To investigate the effect of the drug on the cell cycle distribution analysis described by Krishnan et al. 1975). Western blotting was carried out as described in another paper (Tu et al. 2004). DNA fragmentation was detected by agarose gel electrophoresis. 1×10^6 cells was plated in a 6-well culture plate. When the cells reached approximately 70% confluence, the test samples were added and the cells were incubated for 48 h. After 48 hours, the cells were harvested and pelleted by centrifugation. The harvested cells were washed twice with ice cold PBS. The cell pellet was lysed in a buffer containing 10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, the lysate was centrifuged (13,000x g) for 10 min at 4°C. Then, the supernatant (containing RNA and fragmented DNA, but not intact chromatin) was extracted with phenol-chloroform: isoamyl alcohol (24:25:1) (Sambrook & Russell, 2001). The aqueous phase was brought to 300 mM NaCl and nucleic acids were precipitated with 2 vol of ethanol. The pellet was washed with 70% ethanol, air-dried, and then dissolved in 20 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Following digestion of RNA with RNase A (0.6 mg/ml, at 37 °C for 30 min), the DNA samples obtained were analysed by 2% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide, and visualized as a DNA ladder with UV-trans illumination.

4.2.12 Flow Cytometric Analysis

To investigate the effect of the drug on the cell cycle distribution, the cells (1×10^5 cells/ml) were treated with IC50 concentrations of MET-H-
AuNPs and cultured for 24 h. The treated cells were harvested, washed with PBS and fixed in 75% ethanol at 4 °C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40µg/ml propidium iodide (PI) and 0.1mg/ml RNase A, followed by shaking at 37 °C for 30min (Krishnan et al. 1975). Cells were analysed by flow cytometry (Becton-Dickinson San Jose, CA, USA) and the percentage of cells in different stages were calculated using Win MDI 2.9 software (TSRI, La Jolla, CA, USA).

4.2.13 Western Blotting

Western blotting was carried out as described in another paper (Tu et al. 2004). Briefly, the cells (1.5×10^6) seeded onto 100-mm culture dishes in the presence or absence of different concentration of compounds (IC50 concentrations) were treated for 24h. The medium was removed and the cells were washed with PBS (0.01M, pH 7.2) several times and lysed on ice in lysis buffer containing 100 µg/ml phenyl methyl sulfonyl fluoride (PMSF), 50 mM Tris-base at pH 8.0, 150 mM NaCl, 0.02% NaN3, 1% NP-40, 10µM aprotinin,10µM pepstatin A and 10µM leupeptin. The supernatants were collected by centrifugation at 10,000×g for 5min at 4°C, and were used as the cell protein extracts.

The harvested protein concentration was measured using a protein assay kit (Bio-Rad). Equal amounts of proteins (50-100µg) were separated on 7.5%-12.5% SDS-PAGE gel and electro transferred onto PVDF membrane. Proteins were blocked overnight with 5% non-fat dried milk in PBS-T at 2-8°C. After washing in PBS containing 0.1% Tween 20 three times, the membrane was incubated with the specific primary antibodies in 5% (w/v) skimmed milk in PBST. After overnight incubation at 4°C, the membrane was then washed three times with Tris-Buffered Saline (TBST), incubated further with alkaline phosphatase-conjugated goat anti-
mouse antibody at room temperature for 2 h, and then washed three times with TBST. After reaction with horseradish peroxidase-conjugated goat anti-mouse antibody, the immune complexes were visualized by using the chemiluminescence ECL PLUS detection reagents following the manufacturer’s procedure (Amersham Bioscience). The same nitrocellulose membrane was stripped and incubated with β-actin monoclonal antibody (Sigma) at a 1: 2000 dilution for 1 h, which acted as a control for loading and blotting.

4.3 RESULTS AND DISCUSSION

4.3.1 Characterization of MET-H-AuNPs

In this work, sunlight acts as the radiation energy, the fruit extract acts as a reducing agent and HA acts as a surface coating material for the one pot green synthesis of size-controlled AuNPs. Further, we expect that the synthesized AuNPs would enable attachment of biomolecules for drug delivery applications (Scheme 4.1).

Scheme 4.1 Schematic representation showing Eggplant (Solanum melongena L)-HA reduced and capped AuNPs and subsequent loading of cationic MET on HA capped AuNPs
We observed that the solution containing gold ions (Au$^{3+}$) and (Eggplant + HA) turned into wine red (544 nm) after 20 min of sun light irradiation. We found that 150µL of eggplant extract (Figure 4.1 A) was sufficient to reduce 1mM HAuCl$_4$. FTIR Figure 4.1B (i) spectra peaks at 2925 and 1040 cm$^{-1}$ corresponding to the HA backbone structure functional group of CH and COC. FTIR spectrum Figure 4.1B (ii) of HA reduced Au contains peaks at 1030 cm$^{-1}$indicates the presence of HA functional moieties between HA and AuNPs.

Figure 4.1B (iii) showed the stretching and bending vibrational peaks of NH functional group at 3396 and 1596 cm$^{-1}$. Figure 1B (iv) showed the complexation of HA with MET by the presence of functional groups at 2965 cm$^{-1}$ (CH) and 1596 cm$^{-1}$ (NH). Moreover, the peak appeared at 1645 cm$^{-1}$ (CONH) indicates the formation of amide bond between carboxyl groups of HA and amine group of MET. (Scheme 4.2)

Scheme 4.2 Synthesis of MET-H-AuNPs
Figure 4.1 (A) Optimization of H-AuNPs by changing various concentrations of [egg fruits extract + HA (constant)] while keeping the gold solution as constant. (B) FT-IR study of: (i) HA (ii) H-AuNPs (iii) MET (iv) MET-H-AuNPs

Gunasekaran et al. (2006). Energy dispersive X-ray (EDX) spectrum analysis of H-AuNPs demonstrated the presence of AuNPs in the sample. Figure 4.2(A) reveals a strong and typical optical absorption peak at approximately 1.5 keV, which could be attributed to the SPR of the metallic Au nanocrystals. The selected area of electron diffraction pattern (SAED) of the AuNPs showing the rings designated 1, 2, 3 and 4 arise due to the reflections from (1 1 1), (2 0 0), (2 2 0) and (3 1 1) (Figure 4.2(B)). The lattice planes of the face-centered cubic structure indirectly prove the crystalline nature of AuNPs. Additionally, H-AuNPs
shows multiple crystal domains (Figure 4.2 (C)), with the presence of twin planes (Figure 4.2 (D)) that form at the boundaries between different crystal domains (George et al. 2012). The morphological results (HRTEM) also clearly indicate the crystalline nature of AuNPs. HRTEM images (Figure 4.3 (A)) revealed that the H-AuNPs and MET-H-AuNPs appear to be nearly spherical in shape (Figure 4.3 (B)) with narrow size distribution. The stability of MET-H-AuNPs in different buffers was investigated (Figure 4.3 (C)), and only minimal changes (<10%) were observed in the SPR band at 544 nm when the H-AuNPs were exposed to different buffers (pH 4.5, 6.8, 7.4 and normal saline) for 48 h. However, at pH 1.2, about 24% reduction in the absorbance at 544 nm was observed compared with the absorbance of MET-H-AuNPs at pH 7.4. The observed reduction in the absorption of MET-H-AuNPs in extremely acidic conditions could arise from a limited aggregation of MET-H-AuNPs as a result of the screening of the negative charge on the surface of the AuNPs at pH 1.2.

The synthesized H-AuNPs were stable in different buffers and normal saline mimicking the physiological environment (Kim et al. 2012, Jia et al. 2012). The crystalline structure of nanoparticles was determined based on X-ray diffraction (XRD) analysis. The crystalline peaks that were identified as AuNPs based on XRD analysis showed that the intense peaks of reflected radiation (Bragg peaks) were at the points (11 1), (2 0 0), and (2 2 0), and were the diffraction lines of the face-centered- cubic (fcc) gold shown in Figure 4.2 (B). The selected area of electron diffraction (SAED) outline of the AuNPs showing that the rings designated 1, 2, 3, and 4 arise because of the reflections from (1 1 1), (2 0 0), (220), and (311) shown in Figure 4.2(E).
Figure 4.2 (A) The EDAX spectrum, (B) SAED pattern of eggplant fruit extract + HA reduced H-AuNPs (C) HR-TEM image of a H-AuNPs (5 nm) showing the multiple domains. (D) High magnification showing the twin plane at the boundary of the domains. (E) X-ray diffraction (XRD) spectrum
An insignificant change in the position under pH change and electrolytic conditions indicated greater stability of MET-H-AuNPs. All these results confirm the usefulness of these nanoparticles for drug delivery application. A structural analysis of MET and HA revealed their higher binding affinity, as MET have the positively charged amino group for interaction with the negatively charged HA. As expected, MET could be easily loaded onto H-AuNPs at room temperature within 45 min. MET binding onto H-AuNPs was further confirmed by the shift of SPR band (Figure 4.3 (D))

Figure 4.3 HRTEM image of (A) H-AuNPs (B) MET-H-AuNPs, (C) The UV–vis spectra showing the in-vitro stability of MET-H-AuNPs in the pH range 1.2–7.4 and PBS, (D) H-AuNPs conjugated MET HCL
Towards a higher wavelength (from 544 nm to 556 nm). The percentage of MET loaded onto H-AuNPs was determined based on MET content in the obtained pellet, and it was found that 63±7% of the drug could be loaded. ICP-OES was used to determine the concentrations of gold in the aqueous solutions of H-AuNPs and the same was used in further studies.

4.3.2 Particle Size and Zeta Potential Measurements

The hydrodynamic particle size of H-AuNPs was found to be 30±2 nm with a polydispersity index of 0.230 [Fig.4.4 (i)]. The average hydrodynamic diameter of MET-H-AuNPs is 55±3 nm with a polydispersity index of 0.210 (Figure 4.4 (ii)). A low polydispersity index indicates the uniformity of particle size distribution for H-AuNPs and MET-H-AuNPs. Zeta potential is an essential parameter to study the state of the nanoparticle surface and predict the long-term stability of the nanoparticles. (Zhou, Wang et al. 2009).

The zeta potential of H-AuNPs was found to be −33.2 mV (Figure 4.4 (iii)) and it indicates the AuNPs were properly capped with anionic HA. MET-H-AuNPs were isolated by centrifugation and then suspended in aqueous solution to measure the electrophoretic mobility. The calculated zeta potential of MET-H-AuNPs was −15.9 mV (Figure 4.4 (iv)). Though the surface charge is reduced to some extent, MET-H-AuNPs has enough repulsive force to prevent aggregation during long-term storage.
Figure 4.4 (i) The particle size distribution of H-AuNPs (ii) MET-H-AuNPs. (iii) The zeta potential distribution of H-AuNPs in aqueous medium. (iv) The zeta potential of distribution MET-H-AuNPs in aqueous medium

4.3.3 Toxicity of H-AuNPs Nanoparticles in Zebra Fish Embryos

The developing zebra fish (Danio rerio) embryos had been used towards research on the toxicity testing of AuNPs, Owing for their inherent benefit being a useful model for the research (Kim et al. 2013). The survival rate was expressed as the number of embryos that had hatched by 96 h post-fertilization, as compared with the control group (Figure 4.5A (i)). The mortality level was indicated by the dead embryos 96 h post-fertilization as compared to the control group (Figure 4.5A (ii)). Both
survival rate (%) and mortality (%) studies revealed that there is no apparent toxicity at various concentrations ranging from 50 to 450 µg/ml (Albanese et al. 2011). The H-AuNPs treated embryos were observed using a stereo microscope to find any abnormality such as pericardial edema, pericardial sac of the larvae and tail deformity. Exposure of zebrafish egg embryos to H-AuNPs did not produce any major toxicity in the normal development of the zebrafish (Figure 4.5A (iii)). Hence, this investigation strongly suggests that the H-AuNPs could be a carrier for biomedical and drug delivery applications.

4.3.4 Release profile of MET-H-AuNPs

The drug release profiles of the MET-H-AuNPs and free MET in PBS at 37 ºC are presented in Figure 4.5 (B). pH 7.4 and 5.7 were selected for drug release because they are close to the physiological and endosomal pH conditions of a cancer cell, respectively. The pH-dependent drug release is found to be the most important factor in cancer therapy. The pH of normal tissue is different as compared to that of pathological tissues such as cancerous and infected tissues (Li Chen et al. 2014). As can be seen in Figure 4.5 (B), the MET-H-AuNPs displays the slow and controlled release of the drug, with the release rate calculated to be two times lower than that of free MET in acidic and neutral environments, respectively. Furthermore, the release efficiency of the drug from the MET-H-AuNPs was two times higher at pH 5.7 than at pH 7.4. Also, the negligible release of MET from MET-H-AuNPs in pH 7.4 will help to reduce toxicity of MET to the normal tissue since the physiological pH of body is maintained at pH 7.4. (Ganesh Kumar et al. 2014).
Figure 4.5  [A][i] Graph representing the toxicity of H-AuNPs in terms of survival rate (%) of larvae,[ii] Mortality rate (%) analysis of H-AuNPs toxicity, [iii] Examples of zebra fish larvae exposed to control and various concentration of H-AuNPs. Significant differences compared to control are indicated by (P=0.0002) ***p<0.001 and was calculated with One-way ANOVA test. [B] In-vitro release profiles of MET from the MET- H-AuNPs and free MET in phosphate buffer solution (pH5.7 and 7.4) at 37°C
To find out the release model which best explain the MET release pattern the obtained release data was fitted in various kinetics models like zero order (Figure 4.6a), first order (Figure 4.6b) and Higuchi equations (Figure 4.6c). By comparing the $R^2$ values it was found that high linearity was observed for zero order followed by higuchi kinetics. To understand the mechanism of drug release data was fitted in the Korsmeyer peppas equation. The corresponding plot for Korsmeyer peppas (Figure 4.6d) also showed good linearity. The release exponent “n” was found to be 0.590 which falls in range of 0.45 and 0.89. In general if the value of $n > 0.45$ but $< 0.89$ indicates Non-Fickian (anomalous) release. By considering this it can be understood that MET release from MET-H-AuNPs was controlled by more than single mechanism.

![Figure 4.6a Zero order kinetics](image-url)
Figure 4.6b First order kinetics

Figure 4.6c Higuchi kinetics
4.3.5 In-Vitro Cytotoxicity Studies

HepG2 cells were used to study the anti-cancer activity of MET and MET-H-AuNPs by a standard MTT assay (Kisfalvi et al. 2009). The assay was terminated at 48 h and colorimetric determination of cell viability was performed using the ELISA Reader (Qualigens, Bangalore, India). The results of MTT assay for free and loaded MET on HepG2 cell lines are shown in Figure 4.7(A) & (B). Cell viability images were visualized under the inverted microscope after which the images were processed by WSxM software (Horcas et al. 2007). Close observation of these images clearly indicated that MET-H-AuNPs caused more effective cell death than free MET (Figure 4.7(B)). The IC50 value for MET-H-AuNPs was found to be around 4 µg/ml while that for free MET ranged from 10 µg/ml on the HepG2 cells. These results show that the MET-H-AuNPs exhibited better cytotoxic activity than free MET. This could be a result of the variations in uptake profile leading to better activity of MET-H-AuNPs as suggested by
Mohanty et al. (2010). HAs have been widely used as novel drug carriers for target-specificity and long-acting delivery (Luo et al. 2002). They have been employed both as delivery vehicles and angiostatic agents in cancer therapy (Luo et al. 2000). As a drug carrier, a biocompatible linear polysaccharide of HA is highly efficient in targeted delivery to liver tissues with HA receptors such as the hyaluronan receptor for endocytosis (HARE) and the cluster determinant CD44 (Ahrens et al. 2001, Entwistle et al. 1996). This could be due to the fact that HA functionalized AuNPs can effectively deliver the drug to cancer cells (CD44- overexpressing cells) by means of the active targeting. The result reveals that MET-H-AuNPs were internalized to a greater extent within HepG2 cells than the free drug resulting in higher cytotoxic effect. (Lee et al. 2008)

Figure 4.7 (A) In vitro cytotoxicity studies of drug MET and MET-H-AuNPs in HepG2 cell lines. All assays were performed in triplicate and the mean ± standard deviations are shown; (B) images of HepG2 cell lines visualized under inverted microscope. (i) Control, (ii) MET, and (iii) HA (iv) AuNPs (v) MET-H-AuNPs
4.3.6 Apoptosis Study

The acridine orange and ethidium bromide staining experiment was performed to examine the morphological changes; cytotoxic effect, apoptotic changes and whether nuclear condensation had occurred in HepG2 and NIH 3T3 cells treated by MET-H-AuNPs. HA has a high binding capacity with HA receptors such as hyaluronan receptor for endocytosis (HARE) and the cluster determinant receptor (CD-44). To find out the cell specific delivery and uptake of MET-H-AuNPs the apoptosis study was carried out with two different cell lines (HepG2 and NIH 3T3 cells). HepG2 cells (Liver cancer cells) are positive for CD44 receptor and NIH 3T3 (mouse embryonic fibroblast) cell lines lacks CD44 receptor. Figure 4.8.A (iv, v, vi) showed that the MET-H-AuNPs treated NIH 3T3 cells did not produce any significant cell death in terms of red color cells. The HepG2 cells showed the characteristic morphological changes of apoptosis, including shrinking of the cytoplasm and nuclear fragmentation with an intact cell membrane or a contracted nucleus and condensed chromatin fragments (Karthik, Sankar, Varunkumar, Ravi Kumar, 2014) [Fig.4.8.A (ii)]. The number of apoptotic cells increased dramatically after treatment with MET-H-AuNPs in HepG2 cells [Fig. 4.8.A (iii)]. The red color is apoptotic cells (dead cells) and it was revealed that MET-H-AuNPs treatment resulted in a significant increase in apoptosis compared to MET treatment. The control cells [Fig.4.8.A (i)] the nuclei as well as cytoplasm fluoresced uniformly green indicating that they did not undergo any morphological changes.

4.3.7 DNA Fragmentation Assay

DNA fragmentation assay has been analyzed by agarose gel electrophoresis method show a "ladder or fragmented" pattern of DNA and different bp intervals (~1000bp, ~500bp and ~200 bp).
Figure 4.8 (B) Lane 1 was loaded with 1kb ladder. Lane 2, 3, 4 and 5 were loaded with equal amount of DNA extracted from control HepG2 cells, H-AuNPs, MET and MET-H-AuNPs respectively.

Figure 4.8  


B. Gel electrophoresis of DNA of HepG2 cells treated with. Lane 1: 1 kb ladder, Lane 2: Control, Lane 3: H-AuNPs, Lane 4: MET, Lane 5: MET-H-AuNPs
Intact DNA band was observed in control and H-AuNPs treated cells as no DNA fragmentation has occurred. However, DNA fragmentation was observed in the lane treated with MET and MET- H-AuNPs indicating apoptosis in HepG2 cells. A closer look shows that the DNA fragmentation observed in the sample of HepG2 cells treated with MET-H-AuNPs showed more effective cell death as compared to MET.

### 4.3.8 Effect of MET-H-AuNPs on Cell Cycle Analysis

To investigate whether the MET-H-AuNPs affect regulation of the cell cycle, a flow cytometry study was performed. Fig. 4.9.A shows the cell cycle analysis of [i] control, [ii] MET and [iii] MET-H-AuNPs. Control cells after 36 h had 7.245% minimal accumulation of cells in the G2/M phase [Fig.4.9.A (ii)] interestingly, the MET and MET-H-AuNPs produce a 30.21 % and 44.29 % dramatic increase in the G2/M population, respectively. Hence, our study proves that the MET-H-AuNPs induce cell cycle arrest significantly at the G2/M phase in HepG2 cells. (Kim et al. 2014).

### 4.3.9 Western Blot Analysis

Anticancer therapy medicines eradicate cancer cells by triggering completely different caspase-mediated cell death pathways (SathishSundar et al. 2014). MET-H- AuNPs exhibited potent cytotoxic activity in HepG2 cells. The western blot results showed that considerable change occurred in protein concentration directly which is responsible for the apoptosis. Cyclin D1 plays a critical role in the regulation of proliferation by adjusting its expression levels to reflect the proliferative signaling environment for the cell, and by regulating the cell cycle control machinery accordingly (Matsushime et al. 1994); Weinberg, 1995). MET led to a strong reduction of Cyclin D1 protein levels in tumors (Ben Sahra et al.
The results as seen in Fig. 4.9 (B), showed that MET-H-AuNPs were able to inhibit the function of Cyclin D1 and its expression was dramatically diminished and Cyclin E showed a high intense band as compared to H-AuNPs and native MET treated cells.

The functions of cdk-4, cdk-6, and cdk-2-mediated phosphorylation of retinoblastoma (Rb) family of tumor suppressor proteins during apoptosis were also observed (Joseph et al. 2004). MET-H-AuNPs induced apoptosis in cancer cells and this was evident from the reduced expression of anti-apoptotic Cdk-6 and decreased expression of Cdk-4 and Cdk-2 in HepG2 cells. In this study, to analyze the efficacy of CDK inhibition as a means to inhibit proliferation of HCC, b-actin was used as a loading control and it showed similar expression in all lanes. Intrinsic Pathways of western blot studies are shown in Figure 4.9 (B). The Bax protein is a pro-apoptotic member and bcl-2 is an anti-apoptotic member in the Bcl-2 protein family. The formation of heterodimers among these pro-apoptotic and anti-apoptotic proteins of the Bcl-2 protein family might activate and switch off the caspase-mediated cell death method (Sturm, Kohne et al. 1999). Remarkably, the effects of apoptosis inductions are more dependent on the ratio between bcl-2 and bax than on the quantity of bcl-2 alone. In the present investigation MET-H-AuNPs induced down-regulation of Bcl-2 proteins and up-regulation of Bax proteins in HepG2 cells. p53 is a leading switch that coordinates stress signals linked with apoptosis and cell cycle arrest (Hwang et al. 2012) (Figure 4.9 (B)). MET-H-AuNPs induced increase in the levels of p53, the tumor suppressor protein. Caspases, a family of cysteine acid proteases, can be regarded as the key factors in apoptosis (Wang et al. 2010; Fan, Han, Cong, Liang, 2005). Fig.4.9 (B) shows that MET-H-AuNPs treated cells induced cleavage of caspase 9, and caspase 3. Similarly, the cleaved form of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Chang
et al. 2011). Figure 4.9 (B) also showed that PARP proteins cleaved into a fragment after treatment with MET-H-AuNPs. Our studies showed that MET-H-AuNPs contributed to the obvious notable increase in caspase 9, caspase 3 and PARP proteins.

Figure 4.9 A. Effects of MET-H-AuNPs on cell cycle analysis. HepG2 non-small cell liver cancer cells were cultured for 36 h (i) Control (ii) MET (iii) MET-H-AuNPs. B. Western blot illustrating the different protein expression in apoptosis after treatment with MET and MET-H-AuNPs. Lane 1: control, Lane 2: H-AuNPs, Lane 3: MET, Lane 4: MET-H-AuNPs
4.4 CONCLUSION

In conclusion, we have reported one pot green synthesis of size controlled AuNPs by using eggplant extract combined with HA. Further, applicability of these H-AuNPs as a carrier for the delivery of the cationic drug was demonstrated by successful loading of MET onto synthesized H-AuNPs. *In vitro* cytotoxicity (MTT assay) study showed that MET-H-AuNPs effectively inhibited the multiplying of cancer cells by principle of active targeting. The MET-H-AuNPs exhibited good stability in different physiological conditions. A toxicity study in zebra fish embryo model results revealed that no significant malformation occurred ensuring that the MET-H-AuNPs nanoparticles are highly compatible for drug delivery applications. Apoptosis studies were clearly indicated that MET-H-AuNPs pronounced higher apoptotic behavior in HepG2 cells than NIH 3T3 cells. DNA fragmentation study revealed that necrosis in cells was induced by MET-H-AuNPs. Western blotting and flow cytometry techniques indicated that the MET-H-AuNPs induced higher percentage of cell death in HepG2 cells. These results showed that MET-H-AuNPs could be a more promising material for drug delivery in anti-tumor applications.