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# Synthesis and characterization of biocompatible gold nanoparticles stabilized with hydrophilic polymer coated hesperetin drug for sustained drug delivery to treat hepatocellular carcinoma-derived cancer cells

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## ABSTRACT

The biological application of polymer coated gold nanoparticles in the recent chemotherapeutic work on drug delivery as a carrier has been effective for the treatment of cancer. Obviously in the production of pharmaceutical anticancer drugs, the factors like solubility, bioavailability, biocompatibility, limited chemical stability etc., are necessary with a special formulation technology in effective drug delivery. Our aim of the present study was to improve the solubility of the drug and reduce the side effects of chemotherapy drugs. Therefore, we report a simple and efficient method to synthesize a biocompatible nano carrier (polymer functionalized gold nanoparticles) to load the partially soluble drug (Hesperetin). Initially the carrier viz., the polymer functionalized gold nanoparticles (Au- mPEG $_{(5000)}$-SH) was synthesized by reacting the tetrachloroauric acid ($HAuCl_4$) with polymer (O-[2-(3-Mercaptopropionylamino) ethyl]-O'-methylpolyethylene glycol). Then, the anti-liver cancer drug viz., hesperetin (HP) was loaded on to the Au- mPEG $_{(5000)}$-SH to treat the hepatocellular carcinoma. The formation of polymer functionalized gold nanoparticles and drug loaded polymer gold nanoparticles were characterized using UV-VIS spectrophotometer, HR-TEM with EDAX, and DLS with Zeta potential techniques. The *in vitro* drug release was carried out in PBS at pH 7.4 for Au- mPEG $_{(5000)}$-S-HP and compared with the control pure hesperetin (HP). The *in vitro* cytotoxicity was studied using Hep3B human hepatocellular carcinoma cell line and observed that the cellular uptake of Au- mPEG $_{(5000)}$-S-HP was higher when compare with pure drug (HP). These consistent results suggest that mPEG $_{(5000)}$-S-HP functionalized Au nanoparticles could be used as an efficient drug for *in vivo* to treat hepatocellular carcinoma in animals.

**Key words:** Gold nanoparticles, Hesperetin, Drug delivery, Cytotoxicity.

## 1. INTRODUCTION

In the present scenario, nanotechnology is discovering new innovative ideas in the field of biomedical research with the use of biocompatible polymer and metal nanoparticles. The most commonly studied metal nanoparticles like gold, silver, titanium oxide, iorn oxide nanoparticles [1], gold and silver are biologically inert and cause no serious side effects in the biological systems. For the development of suitable nanomedicines, the particle size and size distribution of nanoparticles are important parameters. It determines a number of activities *in vivo* conditions namely the distribution, biological fate, toxicity and the targeting ability of nanoparticle systems [2]. In particular, gold nanoparticles possess biological activities like antioxidant, anti-inflammatory, anti-angiogenesis and anticancer properties [3-4]. Therefore it has been used for the delivery of drugs [5] proteins, peptides and oligonucleotides etc [6-7]. The use of biocompatible polymers like functionalized PEG, PLGA etc are widely used in the nanostructures development made up with metals [8]. Particularly, monolayer-protected AuNPs have recently emerged as an attractive candidate for delivering various therapeutic agents such as drugs, peptides, proteins and nucleic acids to their targets [9]. Further, it is noted that the polymers used for the synthesis of nanoparticles are playing dual role as reducer and stabilizer [10]. From the existing literature, it is observed that the polymer viz., poly (methylhydrosiloxane), poly (N-vinyl-2-pyrrolidone), poly (sodium acrylate), poly (ethylene oxide), poly (vinyl alcohols) and polyethyleninine are used as both reducing and stabilizing agent in the preparation of nanoparticles [11-13]. The major advantage of using a polymer as a stabilizing agent is that it can be used to tailor the nanocomposite properties and also to provide long term stability of the nanoparticles by preventing particle agglomeration [14-15]. Recently, the functionalized AuNPs were synthesized through different stabilizing and capping agents and showed the potential in several applications [16]. In comparison with the other stabilizing agents like surfactants, flavanoids, alkaloids, etc. PEGylation is one of the most commonly used functionalization methods where a layer of PEG is coated on the surface of AuNPs or in conjunction with other molecules such as biotin, peptides or oligonucleotides. K.Esther et al; has developed a hetero-bifunctional PEGlyated AuNPs, where the AuNPs was functionalized with thiol group on one end and cumarin a fluorescent dye on the other [17]. Hence, it is clear that the binding ability of the AuNPs to the cell membrane and the functionalization of

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the polymer on the AuNPs make it to serve as a good drug carrier [18-21].

Natural products represent one of the most enduring approaches in the development of anticancer targeting drug. Historically, natural products from plants have been the basis from traditional medicine systems. According to the World Health Organization (WHO) primary health care are still relied upon the natural products and approximately 80% of the residents of developing countries in the world uses natural products as foods medicines. An example of the dominant role of natural products can be seen in the last 25 years, where the 77.8% of the cancer therapeutic of approved drugs are either natural products or based on natural products, or mimics of natural products [22]. Hesperetin (HP) was chosen as one of the natural product drug with partially water soluble [23]. Hesperetin (3', 5, 7-Trihydroxy-4-methoxy flavanone) with molecular weight about 302.3; it is a flavonoid that exists widely in plants, fruits, flowers, foods of plant origin [24] and abundant in citrus fruits [25]. The drug hesperetin is an important bioactive compound in medicinal herbs and it also has biological and pharmacological activities, such as anticarcinogenic effect, antihypertensive, [26] anti-inflammatory, [27] antioxidant, [28] and lipid-lowering efficacy [29]. Since many antioxidants exhibit antiplatelet [30] and anticarcinogenic effects ,[31] it is possible that hesperetin can also function in a similar way but hesperetin is poorly soluble in water and shows a slow dissolution rate from solid oral forms, thus restricting its use in therapy [32]. This drug was already reported that it shows anti-carcinogenic effects caused cancers (colon and breast) which is strongly supported by an *in vitro* studies [33]. To enhance the efficacy of the cancer therapeutic agent, the use of nanoparticle-based drug formulation is an important aspect of nanomedicine [34].

In this highlight, we aimed to synthesis gold (Au) nanoparticles (NPs) stabilized and reduced with polymer O-[2-(3-Mercaptopropionylamino)ethyl]-O'-methylpolyethylene glycol (mPEG$_{(5000)}$-SH). Further, it is capped with anti cancer drug-hesperetin (HP) for effective drug delivery to treat hepatocellular carcinoma. The Au-mPEG$_{(5000)}$-SH consists of an Au core, inner shell of hydrophilic heterobifunctional methyl polyethylene glycol (mPEG$_{(5000)}$-SH) polymer and outer shell of anti cancer drug hesperetin (HP). From the literature survey, it is clear that there is no report available on gold nanoparticle based delivery of anticancer drug hesperetin for treatment of hepatocellular carcinoma.

The synthesized chemical structure of Au-mPEG$_{(5000)}$-SH and Au-mPEG$_{(5000)}$-S-HP was characterized using UV-Visible Spectroscopy. The morphological studies were carried out using High Resolution Transmission Electron Microscopy (HR-TEM) and Dynamic Light Scattering (DLS) and Zeta potential techniques. In addition, HP was used to evaluate the *in vitro* drug releasing capacity of the Au-mPEG-SH. The cellular uptake and cytotoxicity of the carrier Au-mPEG$_{(5000)}$-SH with drug HP against Hep3B human liver carcinoma cells were assessed using confocal microscope and the MTT assay.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Tetrachloroauric acid ($HAuCl_4$), O-[2-(3-mercaptopropionylamino) ethyl]-O'methylpolyethylene glycol-5000 (PEG-SH), and hesperetin were purchased from Sigma-Aldrich. Culture medium Minimum Essential Medium (MEM) with L-Glutamine, Sodium bicarbonate, and Sodium pyruvate, antibiotics and calf serum were obtained from invitrogen. All the other chemicals used were of analytical reagent grade.

### 2.2. Synthesis of gold mediated polymer coated drug

Gold nanoparticles (AuNPs) have been synthesized using a modified Turkevich technique [35]. We have used Tetrachloroauric acid as a precursor and O-[2-(3-Mercaptopropionylamino) ethyl]-O'methylpolyethylene glycol (mPEG-thiol, MW = 5000 Da) as both reducing agents and stabilizing agent. The reaction was set at room temperature in dark condition. Briefly, in a 50ml beaker flask, add 0.01g of gold ($HAuCl_4$, $1x10^{-3}$M) was made to dissolve in 10ml of deionized water with the help of magnetic stirrer. To this stirring solution, 0.127g of polymer (PEG-SH, $1x10^{-3}$M) was made to dissolve in 15ml of deionized water. This reaction mixture was made to stir for about 30 minutes until the color changes from yellow to pink. This was treated as control (Au-mPEG$_{(5000)}$-SH). Further, to a part of this solution, 0.005g of drug hesperetin ($1x10^{-3}$M) was added in aliquots and made to stir for about 3 hours. The appearance of pale pink color confirms that the drug is loaded on the surface of the polymer functionalized gold nano (Au-mPEG$_{(5000)}$-S-HP) is as shown in figure.1. Finally the solution was filtered through a 0.45 µm filter paper.

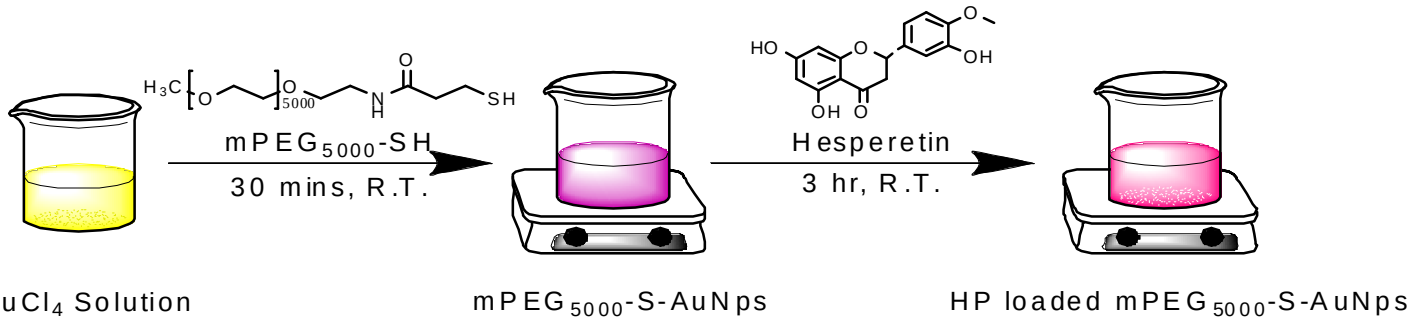



**Figure 1: Schematic illustration for the chemical synthesis of mPEG$_{(5000)}$-SH polymer stabilized gold nanoparticles and hesperetin (HP) loaded mPEG$_{(5000)}$-SH stabilized gold nanoparticles.**

### 2.3. Characterization of gold nanoparticles

The SPR peaks of the AuNPs were characterized with UV-Vis Spectroscopy using Shimadzu UV-1601 spectrophotometer at the wave length of 400 to 800nm. The high resolution transmission electron microscopy was carried out using FEI TECNAI G2 model T-30 at accelerating voltage of 200KV to capture the images. The samples for HR-TEM imaging was prepared by placing a drop of gold solution on a carbon coated copper grid and drying at room temperature. The Dynamic light scattering (DLS) and Zeta potential of the samples were determined by Zetasier nano ZS (Malvern Instruments, UK). A He-Ne diode laser (633nm) as the source was scattered at a fixed angle of $90^0$ C at room temperature. The experiments were performed in triplicates.

### 2.4. *In vitro* drug release study

The invitro drug release from the Gold Nanoparticles loaded hesperetin and pure hesperetin was studied in phosphate buffered saline solution (PBS) at pH 7.4. Au-mPEG$_{(5000)}$-S-HP micelles (4mg/ml), dispersed in PBS solution, were sealed in dialysis bag (MW cutoff: 12-16 kDa), and incubated in the release medium (25ml) at 37°C under oscillation at 90 r min$^{-1}$. To measure the drug release content, samples (1ml) were periodically removed and replaced with an equivalent volume of the phosphate buffer solution. The amount of pure hesperetin and Gold nanopartilces loaded hesperetin was analyzed with a spectrophotometer at 289 nm in triplicate.

### 2.5. Cellular uptake and cytotoxicity studies

The cytotoxicity of Au-mPEG$_{(5000)}$-S-HP and pure hesperetin (HP) against Hep3B cell was assessed using MTT assay [36]. The Hep3B cells were plated in 96 well plates at a concentration of 5 x $10^4$ cells/well. After 24 h, cells were washed twice with 500µl of serum-free medium and starved by incubating the cells in serum free medium for an hour at 37°C. After starvation, cells were treated with Au-mPEG$_{(5000)}$-S-HP and pure hesperetin (HP) of different concentrations for 48 hours. At the end of treatment, the medium from control and Au-mPEG$_{(5000)}$-SH-HP, and HP (hesperetin) treated cells were discarded and 500µl of MTT containing MEM medium (0.5mg/ml) was added to each well. The cells were then incubated for 4h at 37°C in the $CO_2$ incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS (1ml). The crystals were then dissolved by adding 500 µl of solubilization solution and this was mixed properly and the spectrophotometrical absorbance of the purple blue formazan dye was measured in microplate reader at 620 nm. The OD of each sample was then compared with the control OD and the graph was plotted.

## 3. RESULTS AND DISCUSSION

### 3.1. UV-Visible spectroscopy

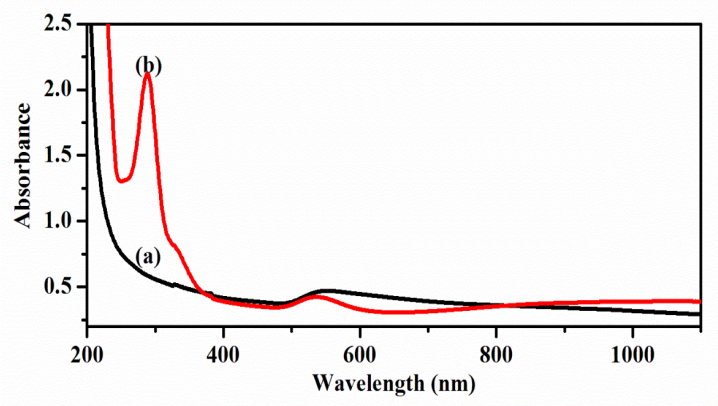
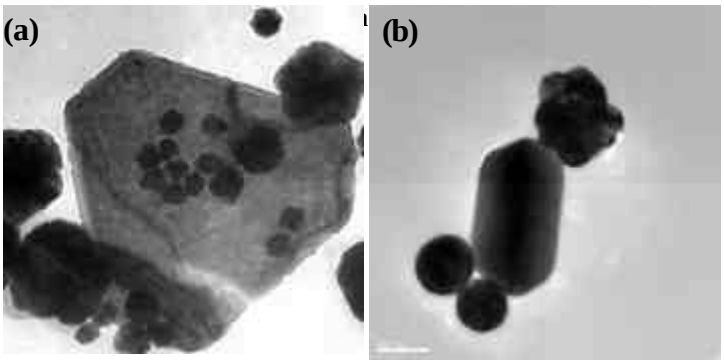



**Figure.2. UV-visible absorption spectra of (a) mPEG$_{(5000)}$-SH functionalized gold nanoparticles and (b) hesperetin (HP) loaded mPEG$_{(5000)}$-SH gold nanoparticles.**

The UV-Visible spectra of Au-mPEG$_{(5000)}$-SH and HP loaded Au-mPEG$_{(5000)}$-SH nanopraticles were showed in figure. 2. The characteristic SPR peak at 552 nm and 537 nm confirms the formation of gold nanoparticles as shown in figure.2. (a) and 2 (b) respectively. The peak at 552 nm was observed immediately after the addition of mPEG$_{(5000)}$-SH polymer to the auric chloride solution. This clearly confirms that the formation of gold nanoparticles is achieved without any addition of external reducing agent [37-38].Consequently, on the addition of the drug hesperetin to this polymer stabilized gold nanoparticles (Au- mPEG$_{(5000)}$-SH) the SPR peak shift to 537 nm. This shift from 552nm to 537nm conforms that the drug is loaded on the surface of Au-mPEG$_{(5000)}$-SH to obtain HP loaded Au-mPEG$_{(5000)}$-SH. In addition the obtained peak observed at 288 nm in figure. 2 .(b) corresponds to the hesperetin drug which in turn the drug physically binded through week van der walls force of interaction without undergoing any chemical changes. That it is clear that the drug is loaded on the surface of the polymer stabilized gold nanoparticels and are facile for the release at the target site.



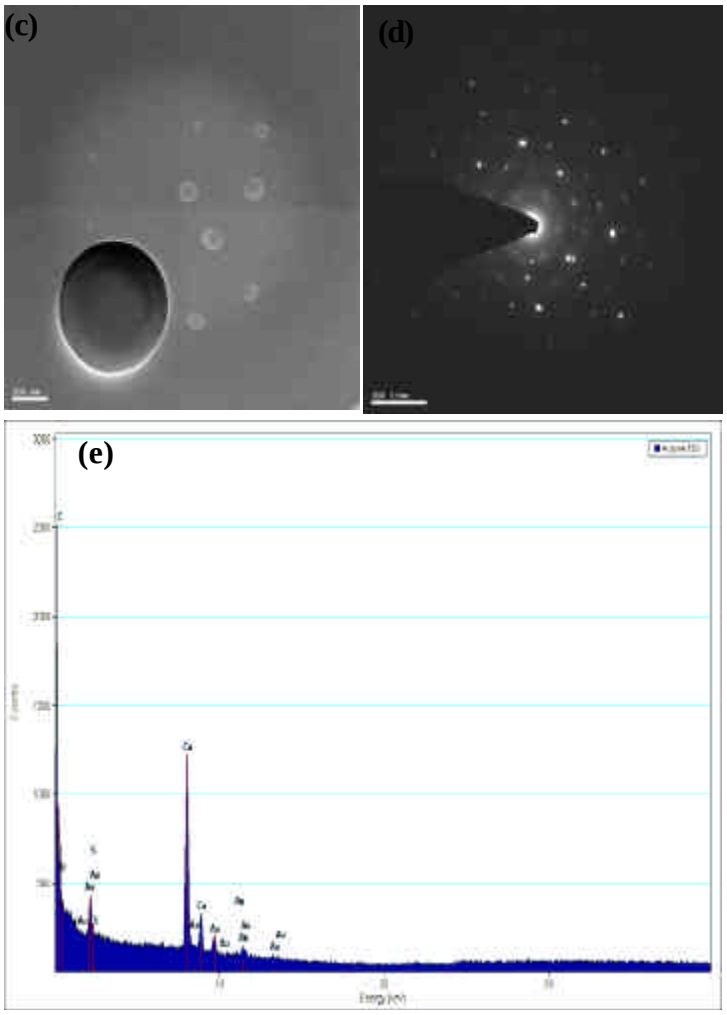



**Figure.3. HR-TEM image of (a), (b) and (c) shows the different shape and size of gold nanoparticels stabilized with polymer (mPEG$_{(5000)}$-SH) and (d) the selective area energy dispersion (SAED) pattern showing that the nanoparticles are crystalline and (e) the EDAX spectra of the gold nanoparticles.**

To determine the surface morphology and size of the Au- mPEG$_{(5000)}$-SH and Au-mPEG$_{(5000)}$-S-HP nanoparticles, we relied on High Resolution Transmission Electron Microscopy technique to capture the images. The HR-TEM image of gold nanoparticles stabilized using mPEG$_{(5000)}$-SH were shown in figure.3 (a), 3 (b) and 3 (c). From the images it is observed that the gold nanoparticles are spherical, triangular and pentagon in shape. This is in accordance with the characteristic shape of the gold nanoparticle such as rod, spherical, triangular, pentagon, hexagon, etc.[39]. From the figure. 3(a), 3(b) and 3(c) the average size of the nanoparticels is 220 nm and from the figure. 3(c) the size of the gold nanoparticels which is embedded in the thiol functiolized poly ethylene glycol polymer with diameter 300 nm that is, t hey form a core shell type where the gold nanoparticle forms the core which is surrounded by the polymer coating thus stabilizing the nanometal and preventing from agglomeration. From the figure 3 (d) the selective area energy dispersion (SAED) pattern showing that the nanoparticles are crystalline in nature.

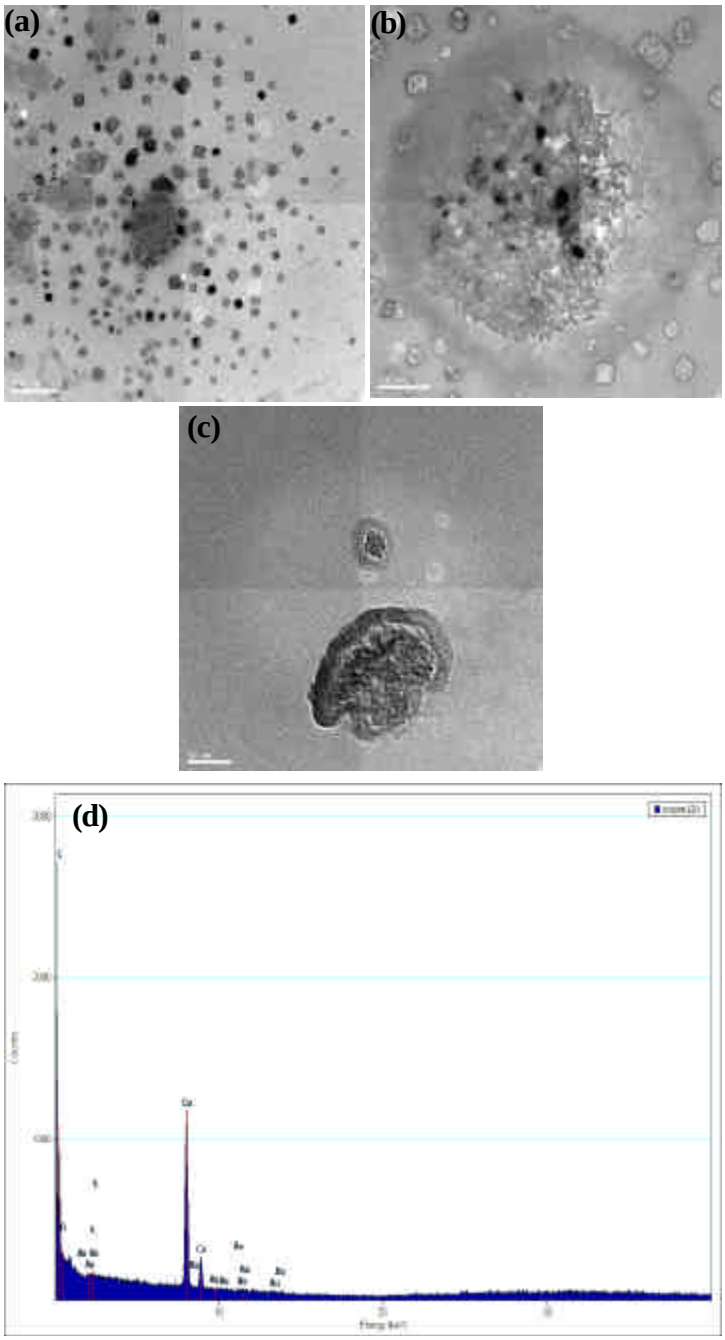



**Figure.4. HR-TEM image of Gold Nanoparticles stabilized with polymer and capped Drug (Au-mPEG$_{(5000)}$-S-HP).**

The HR-TEM image of hesperetin loaded gold nanoparticles stabilized polymer (Au-mPEG$_{(5000)}$-S-HP) was as shown in figure. 4. The external morphology of Au- mPEG$_{(5000)}$-S-HP was shown in figure 4 (a) on focusing in to the single rectangular crystal and the image captured as shown in figure 4 (b) a spherical inner and outer core in both the figure. 4 (a), (b) and (c), that corresponds to the hesperetin loaded gold nanoparticles with 110-120 nm in size respectively. When compare with the control (Au-mPEG$_{(5000)}$-SH), the size of the

nanoparticles will be reduced due to the addition of the hesperetin. Since flavonoid, it has the properties of reduction of gold ions [40]. Huang et al. reported that various polyols and terpenoids were responsible for the generation and stabilization of NPs and its play the major role in bioreduction [41-42].

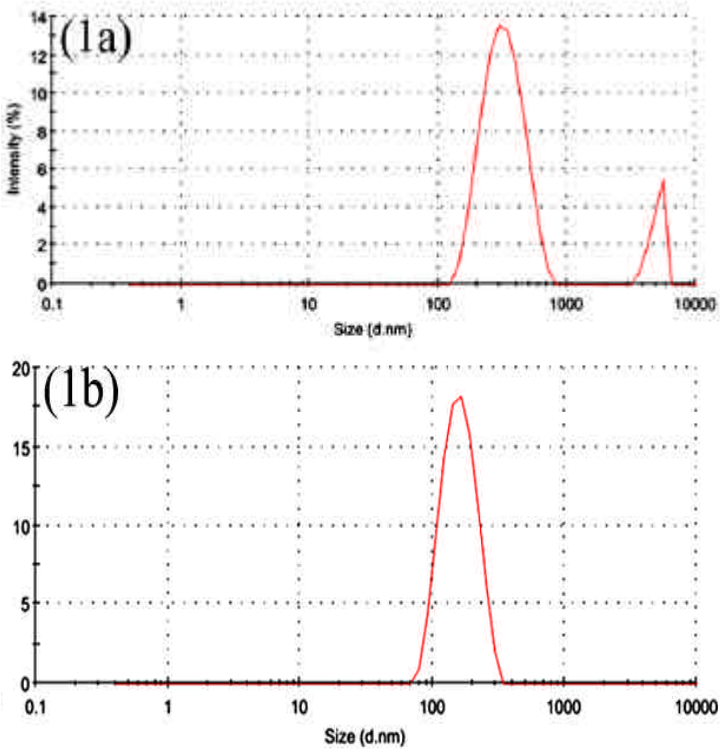
The EDAX spectrum of the above said two product viz, Au-mPEG $_{(5000)}$-SH and Au-mPEG $_{(5000)}$-S-HP was shown in figure. 3 (e) and 4 (d) respectively. From the spectra 3 (e), the presence of gold in addition to the Carbon, Oxygen and Sulphur elements is observed. Further, from the figure. 4 (b), it is clear that the same elements are present. While studying about the percentage composition of each elements as shown in table 1. The percentage composition of carbon and oxygen alone increases in drug loaded polymer AuNPs. This confirms that the drug HP is loaded on the polymer coated gold nanoparticles.

**EDAX percentage composition of elements**

**Table.1. The percentage composition of Elements presents in Au-mPEG $_{(5000)}$-SH and Au-mPEG $_{(5000)}$-S-HP.**

| | % Composition | |
|---|---|---|
| **Elements** | **Au-mPEG $_{(5000)}$-SH** | **Au-mPEG $_{(5000)}$-S-HP** |
| **Carbon** | 59.52 | 64.28 |
| **Oxygen** | 22.60 | 26.19 |
| **Sulphur** | 10.27 | 4.76 |
| **Gold** | 10.27 | 4.76 |

### 3.3. Dynamic light scattering measurement and Zeta Potential



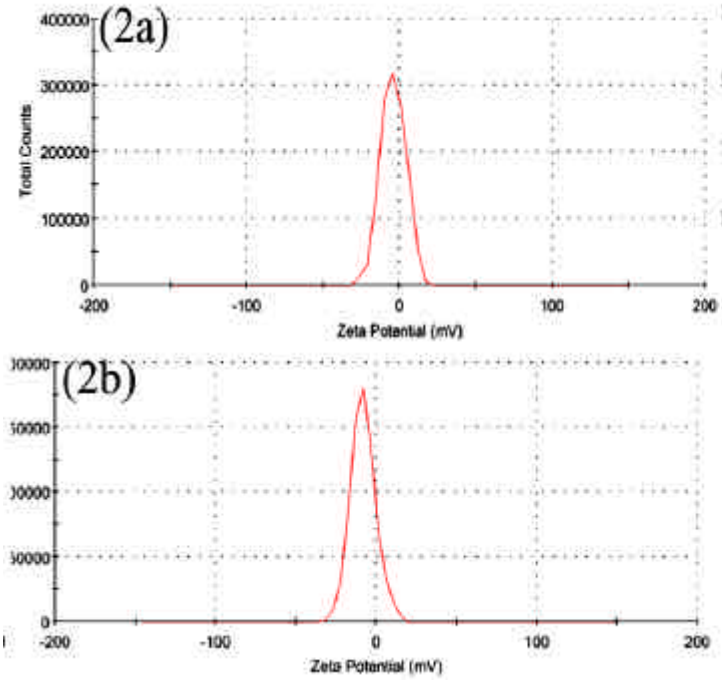
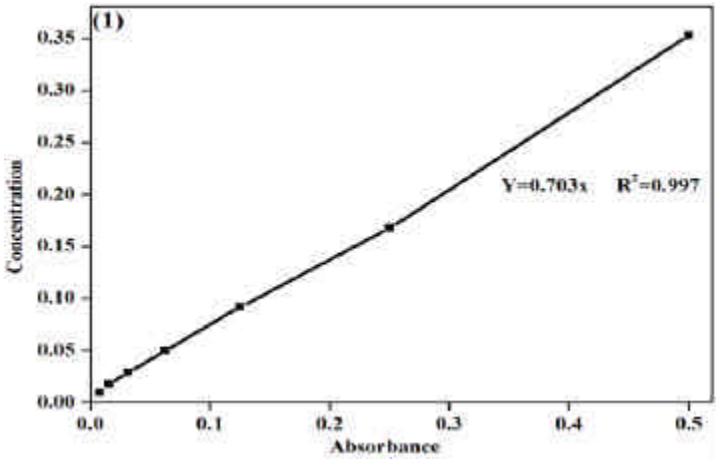




**Figure.5. DLS (1a&1b) and Zeta potential distribution (2a& 2b) graph of Au-mPEG $_{(5000)}$-SH NPs and Au-mPEG $_{(5000)}$-S-HP NPs.**

The size of Au-mPEG $_{(5000)}$-SH and Au-mPEG $_{(5000)}$-S-HP nanoparticels were determined using DLS and Zeta Potential. As shown in figure .5. (1a) the size distribution graph of Au-mPEG $_{(5000)}$-SH narrow area peaks shows the homogeneity of the nanoparticles formed and the average diameter of Au-mPEG $_{(5000)}$-SH NPs is 320 nm.The size difference between the HR-TEM results and zeta potential characterization is due to the hydration of nanoparticles in solution in zeta potential analysis [43]. The figure. 5. (1b) peak shows the nanoparticles formed and the average diameter of Au-mPEG $_{(5000)}$-S-HP NPs is 150 nm. Likewise the Zeta potential distribution of figure 5. (2a) Au-mPEG $_{(5000)}$-SH NPs shows negative charge greater than -4.38mV and the figure (2b) Au-mPEG $_{(5000)}$-S-HP NPs shows negative charge greater than -8.42mV.

### 3.4. *Invitro* Drug release



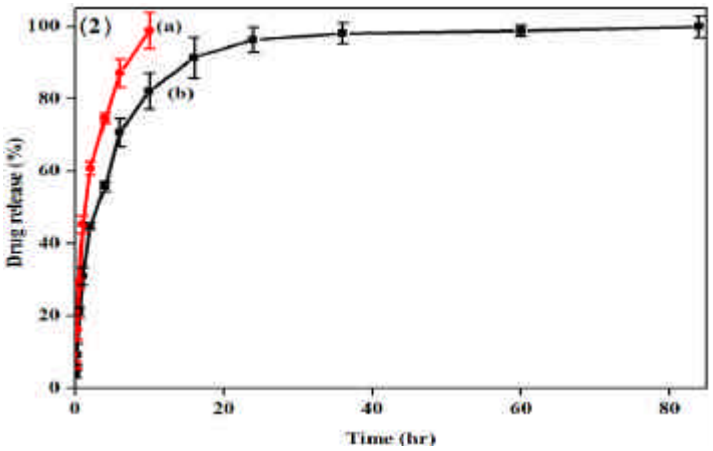



**Figure.6. *Invitro* release study of (1) hesperetin (HP) from (2.a) pure hesperetin (HP) suspension and (2.b) hesperetin loaded goldnanoparticles (Au-mPEG $_{(5000)}$-S-HP NPs) at different time points. Values are mean ± SEM (n=3).**

The *invitro* drug release profile of Au-mPEG $_{(5000)}$-S-HP NPs was illustrated in figure 6 (2) as compared with standard graph figure. 6 (1) of hesperetin drug. As shown in figure 6 (1) showed that pure hesperetin (HP) was released to the extent of 99% within 8 h, the same as reported in Kuntal Maiti etal; [44] when compare with the pure hesperetin drug figure. 6 (2.a), shows the sudden release within 10 h where as in figure 6 (2.b) Au-mPEG $_{(5000)}$-S-HP NPs can be sustained and persisted release for 72 h. The result produced by the hesperetin loaded AuNPs may be a combined effect of sustained release property. It shows that more than 80% of HP was released from AuNPs for 72 h suggested the potential of the nanoparticles as a sustained drug delivery system. Other antidiabetic drugs e.g. rosiglitazone loaded gelatin nanoparticles have similar sustained release behavior from drug loaded nanoparticles [45]. These results indicated that the Au-mPEG $_{(5000)}$-S-HP NPs could be a good candidate for drug carriers.

### 3.5. Cellular uptake and cytotoxicity of pure HP and Au-mPEG $_{(5000)}$-S-HP NPs

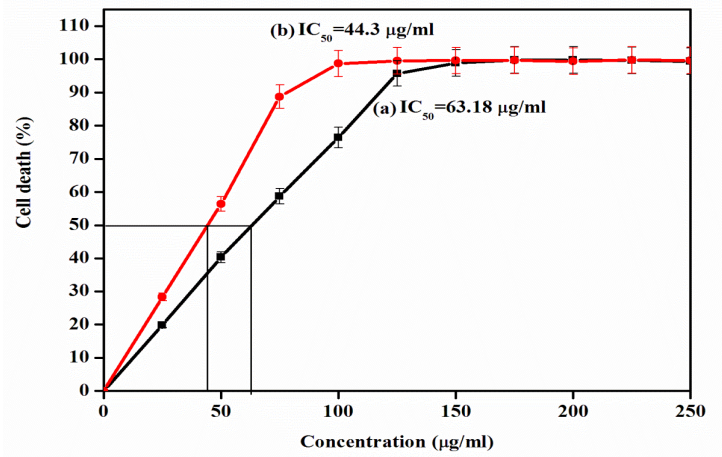
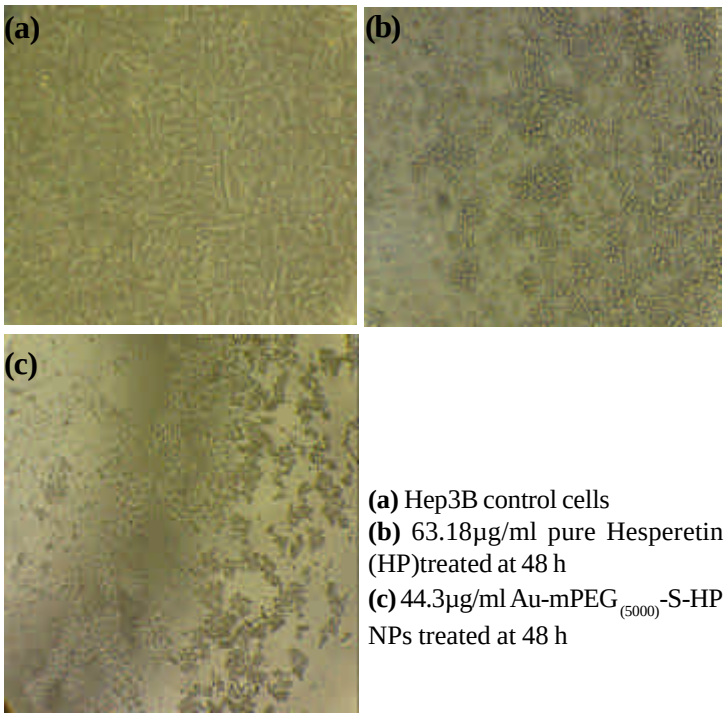



**Figure.7. Effect of (a) Pure hesperetin (HP) and (b) hesperetin loaded gold nanoparticles (Au-mPEG $_{(5000)}$-S-HP NPs) at various doses on Hep3B cells for 48 h as assessed by MTT assay**

The cytotoxic effect of pure hesperetin (HP) and hesperetin loaded polymer coated gold nanoparticles (Au-mPEG $_{(5000)}$-S-HP NPs) was estimated by cell viability measurement as shown in figure 7. The effect of pure hesperetin (HP) and hesperetin loaded gold nanoparticles (Au-mPEG $_{(5000)}$-S-HP NPs) at different doses (10µg/ml-250µg/ml) on Hep3B cells for 48 h by MTT assay. Pure hesperetin and hesperetin loaded gold nanoparticles (Au-mPEG $_{(5000)}$-S-HP NPs) were inhibited the growth of Hep3B cells in a dose dependent manner. The $IC_{50}$ value of pure hesperetin is 63.18 µg/ml and the $IC_{50}$ value of when compare with Au-mPEG $_{(5000)}$-S-HP NPs is 44.3 µg/ml. Based on this study we fixed the optimum doses of Au-mPEG $_{(5000)}$-S-HP NPs as 44.3 µg/ml at 48 h.




**(a)** Hep3B control cells
**(b)** 63.18µg/ml pure Hesperetin (HP)treated at 48 h
**(c)** 44.3µg/ml Au-mPEG $_{(5000)}$-S-HP NPs treated at 48 h

**Figure 8. Morphological changes of Hep3B cells analyzed under phase contrast Microscopy (20x)**

Figure.8. Indicates the Control, pure hesperetin (HP) and hesperetin loaded polymer coated gold nanoparticles cells with clear and unclear form of monolayer under a phase contrast microscope. It exhibit the typical carcinoma type morphology with a clear uniform layer (figure. 8a). After the incubation for 48 h of Hep3B cells with pure hesperetin (HP) is observed to be 63.18µg/ml and hesperetin loaded polymer coated gold nanoparticles (Au-mPEG $_{(5000)}$-S-HP NPs) to be 44.3µg/ml. Hesperetin showed remarkable morphological alterations (figure. 8b and c). From the figure. 8 b and c it is obvious that hesperetin loaded polymer gold nanoparticles treatments resulted in decrease in number of cells and the number of floating cells were found to be increased.

### 4. CONCLUSION

In conclusion, the present study demonstrates that the hesperetin capped polymer coated gold naoparticles have been synthesized by

a wet chemical process. The synthesized Au-mPEG $_{(5000)}$-S-HP NPs showed small size, narrow distribution and relatively good biocompatibility and moreover it shows very good solubility than the pure drug. The polymer (mPEG $_{(5000)}$-SH) act as both reducing agent as well as stabilizing agent where it confirms in all characterization reports. As in the drug delivery result, more than 80% of HP was released from AuNPs for 72 h suggested the potential of the nanoparticles as a sustained drug delivery system. The cytotoxicity of Au-mPEG $_{(5000)}$-S-HP NPs to Hep3B cells was higher than that of pure hesperetin, indicating that the hesperetin loaded gold naoparticles have the ability to treat the cancer cells. The microscopy results were similar to that of MTT assay. These results indicated that the Au-mPEG $_{(5000)}$-S-HP NPs could be a promising anticancer nanomedicine to achieve better efficacy for chemotherapy to treat hepatocellular carcinoma. The solubility, biocompatibility and bioavailability of hesperetin loaded polymer coated gold nanoparticles (Au-mPEG $_{(5000)}$-S-HP NPs) was greatly improved compared to uncoated goldnanoparticles this is because due to the properties of the polymer. Based on these results, hesperetin loaded polymer coated gold nanoparticles appear to be non-cytotoxic while retaining the optical properties advantageous for *in vivo* biomedical applications. Therefore, there is good basis upon which to test the safety, bio distribution, solubility and efficacy of hesperetin loaded polymer coated gold nanoparticles *in vivo* and fully explores their *in vivo* use as efficient drug to treat hepatocellular carcinoma in future studies.

## 5.ACKNOWLEDGEMENTS

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