ANALYTICAL DETECTION AND BIOLOGICAL ASSAY OF ANTILEUKEMIC DRUG USING GOLD NANOPARTICLES

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Introduction

Materials in the nanometre size range may possess beneficial properties which can influence the development of remarkable industrial and engineering applications including biotechnological systems. The formation of hybrid systems comprising nanoparticles and biomolecules paves the way from cell markers to biosensing^{1,2}, bioimaging³ and targeted drug delivery^{4,5}. Nanoparticles have been found to be useful in the development of systemic, oral, pulmonary, transdermal and other administration routes to study drug targeting, the enhancement of drug bioavailability and protection of drug bioactivity and stability⁶⁻⁸. For instance, nanoparticles can be used to enhance oral delivery^{6,9} by improving the bioavailability of poorly absorbed drugs. They are able to penetrate the cells to facilitate cellular internalization and connective tissue permeation, thus enabling the drugs to be delivered efficiently to the targeted tissue without clogging capillaries^{10,11}. Consequently, nanotechnology is now used as potential route to study the delivery of anti-leukaemic drugs and used to improve drug diffusion through the blood/brain barrier. Nanoparticles can also act as drug carriers during intravenous injection, which allows the carriers to penetrate the membranes of the cells and deliver the drugs to cancerous tumours. A recent literature review has indicated that some nanoparticle-bound antitumour agents showed prolonged drug retention in tumours, reduction in tumour growth and prolonged survival of tumour bearing animals¹²⁻¹⁵. Optical¹⁶ and electrochemical¹⁷ sensing of biomolecules using colloidal gold is widely studied by the conjugation of various biomolecules such as protein A, avidin, streptavidin, glucose oxidase, horseradish peroxidase and immunoglobins, *etc.*.

6-Mercaptopurine (6-MP) has achieved a special place in biomedicine, owing to its activity in cancer chemotherapy²¹. It is one of the oldest antineoplastic drug currently in use and as there is a good correlation between the drug's coordination chemistry (it contains sulphur and nitrogen donor sites with potential for binding at *N*-1, *N*-3, *N*-7 and *N*-9) and its chemotherapeutic activity, it has attracted much attention²²⁻²⁴. The activity of 6-MP on tumour cells is believed to be due to its ability to convert these into the corresponding ribosides. In order to suppress the undesirable side effects associated with anti-tumour drugs, metal-drug complexes are currently used as slow release prodrugs of 6-MP. The latter employ coordinated components of certain transition metals like platinum and palladium²⁵. A number of detection methods have been suggested for the identification of 6-MP, which include potentiometric titration for tablet assay²⁶, amperometric detection at a phthalocyanine-modified electrode²⁷ and spectrophotometry²⁸. Whilst Zima *et al.*²⁹ reported the determination of 6-MP using fast polarography and differential pulse polarography at the dropping mercury electrode (DME), Basina *et al.* studied the electrochemical behaviour of 6-MP using catalytic cathodic stripping voltammetry³⁰ and found that 6-MP-modified mercury electrodes act as promoters for the redox exchange of Cytochrome C.

In the present work, gold nanoparticles have been used as probes for the detection of the anticancer drug 6-MP and evidence for their complexation has been obtained using UV-Visible spectroscopy, cyclic voltammetry, transmission electron microscopy (TEM), and fluorescent spectroscopy. Furthermore, the efficacy of the complexes of 6-MP and colloidal gold is examined with biological assays in order to evaluate their antibacterial and antifungal activities.

Experimental

Apparatus

Samples were characterized by ultraviolet (U.V.)-visible spectroscopy (Perkin-Elmer Lambda 25). The path length was 1 cm and matched 1 x 1 cm² cuvettes were used. Transmission electron microscopy (TEM) was undertaken employing a Tec NIE 10 instrument with an accelerating voltage of 120 kV; samples were prepared by mixing aqueous solution of MP (5mM) and gold solution (1mM). Fourier transform infrared (FTIR) spectroscopy was performed using a PE IR

SPECTRUM ASCII PEDS 1.60 spectrometer and samples were presented as KBr pellets. Spectra were acquired at room temperature at a resolution of 4 cm^{-1} . Optical emission spectroscopy was carried out using Fluoromax-2, Gram 386 spectrometer. Cyclic voltammetric studies was performed using an Autolab PG Stat 12 electrochemical analyzer in a three electrode cell, using platinum wire as the counter electrode and a 50 mM KCl calomel reference electrode. The scans were initiated at -0.4 V and all electrochemical measurements were performed in a static nitrogen atmosphere. The working electrode used in this work was indium-tin-oxide (ITO) coated with colloidal gold and this was prepared in the following manner. The ITO slides were cleaned thoroughly by ultrasonication in water, followed by acetone. The cleaned glass slides were immersed in a solution (2% v/v) of 3-aminopropyl trimethoxysilane (APTMS) in methanol for 20 hours and subsequently immersed in freshly prepared colloidal gold for another 15 hours to create a submonolayer of gold nanoparticles. For electrode modification, freshly prepared gold plates were immersed for 12 hours in 6-MP solution (1 mM in 0.01 M HClO_4), to avoid sulphide contamination. This process was repeated for all the electrochemical studies (Scheme 1).

Insert Scheme 1

Materials

$\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (98 %) and trisodium citrate (99 %, AR) were purchased from CDH and Analytical Rasayan respectively. 6-Mercaptopurine (6-MP) was synthesized according to a modified, previously reported procedure^{32,33} and the purity of the sample ascertained by comparing it with the commercial sample obtained from Aldrich. The reaction scheme is given below (Scheme.2)

Insert Scheme 2

Synthesis of 6-chloropurine

To phosphoryl chloride (200 cm^3 , 2.2 mol.) was added drop wise water (20 cm^3) and, after all the water had been added, the mixture was boiled for 1.5 hours to dispel the hydrogen chloride. The mixture was subsequently cooled and the top layer was used for the chlorination of hypoxanthine to form 6-dichloropurine. A mixture of hypoxanthine (8 g) and pyrophosphoryl chloride (64 cm^3) was heated in a sealed glass tube at $165\text{ }^\circ\text{C}$ for 19 hours. After cooling, the brown solution was

decanted from the solid residue in the tube and the volatile material was removed under reduced pressure. The syrupy residue was poured on to crushed ice (200 g), a small amount of tan precipitate removed and the filtrate was repeatedly extracted with portions of diethyl ether (6 x 350 cm³). The ethereal solution was allowed to stand over anhydrous potassium carbonate for one hour and then over calcium sulphate overnight. On evaporation of diethyl ether, the crude product (4.3 g., 43 %), m.p. 175-177 °C, was obtained. A small portion was recrystallized from boiling water (150 cm³) and was allowed to stand at 0 °C for 1.5 hours and then filtered through a sintered glass funnel.

Preparation of 6-mercaptopurine (6-MP)

A suspension of 6-chloropurine (0.98 g, 6.3 mmol.) and an equimolar quantity of thiourea in absolute ethanol (14 cm³) was heated to reflux; the solids dissolved and soon a yellow crystalline product precipitated. After refluxing for one hour, the mixture was chilled and crude 6-MP (0.64 g) was collected, the product was dried over P₂O₅ *in vacuo* at 100°C.

Preparation of citrate capped gold nanoparticles

HAuCl₄.6H₂O (1 mM, 500 cm³) was heated and to the boiling solution was added trisodium citrate (38.8 mM, 50 cm³) as one portion. After the addition, the previously yellow solution of gold chloride turned wine red in colour and gave a characteristic absorbance at 518 nm in the U.V-Visible spectrum. From the TEM measurements, the average diameter of the gold nanoparticles was found to be in the range 17-18 nm.

Preparation of 6-mercaptopurine (6-MP) coated gold nanoparticles

Citrate-stabilized gold nanoparticles (1mM, 50 cm³) were mixed with 6-MP (5 mM) in 2-propanol (25 cm³) and stirred effectively for 5 hours until the wine red colour became blue. The 6-MP coated gold nanoparticles were obtained after centrifugation.

Details of Microbial Assay

Antibacterial and antifungal activities were studied using a disk diffusion method, wherein a suspension of both gramme positive and gramme negative organisms were added to sterile nutrient

agar at 45 °C and the mixture was solidified on a Petri dish. Disks made from filter paper dipped in 6-MP and 6-MP-gold were placed on agar plates and the plates were left for one hour at 25 °C to allow a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. The plates were again incubated, this time at 37 °C for 24 hours, and observed for antibacterial activity by determining the diameters of the zones of inhibition for each of the samples.

Results and Discussion

The plasmon band observed for the wine red colloidal gold at 518 nm in the U.V-Visible spectrum is characteristic of gold nanoparticles. Pure drug shows a maxima at 321 nm. The addition of 6-MP to colloidal gold results in a reduction in the intensity of this absorption band at 518 nm and 321 nm corresponding to Au(0) and drug moiety and is accompanied by the emergence of an additional peak at 670 nm (Figure 1).

Insert Figure 1

The latter can be verified by a colour change from purple to blue with the addition of drug to colloidal gold. The time dependent UV-Visible spectra were obtained after mixing (0.5 mM) 6-MP with gold nanoparticles at timed interval of 30 minutes (curves a-d); curve (e) was obtained after 5 hours. The appearance of a new peak is due to the aggregation of gold nanoparticles and the replacement of citrate by 6-MP leading to the formation of gold-drug complex.

These observations are in agreement with the data obtained using FT-IR spectroscopy. 6-MP exists as tautomer form in the solid state as C=S group and the IR spectrum of free (uncomplexed) 6-MP (Figure 2) displays absorption bands at 1275 cm^{-1} due the presence of the C=S group.

Insert Figure 2

This peak was absent in the spectrum of the adsorbed form (Figure 3) apparently confirming the existence of complex formation of the 6-MP through the sulphur atom.

Insert Figure 3

Fluorescence studies were also undertaken to ascertain the binding of the drug moiety with the gold nanoparticles. Thus, free 6-MP has a broad emission centred at *ca.* 380 nm when excited at a wavelength of 320 nm. The non-fluorescent gold nanoparticles are transformed into a markedly fluorescent species when added to 6-MP, the intensity of fluorescence being decreased when colloidal gold is added to the drug (Figure 4).

Insert Figure 4

Insert Figure 5

The fluorescence spectrum of 6-MP was studied with different concentrations of gold nanoparticles (Figure 6).

Insert Figure 6

With a gradual increase in concentration of Au(0), (Figure 6b-e), the intensity of 6-MP (Figure 6) decreases concomitantly. Throughout this experiment the concentration of 6-MP remained constant and displayed peaks at 424 nm and 368 nm in the emission and excitation spectra respectively. Cyclic voltammetry (cv) was employed using gold nanoparticle-modified electrodes, to ascertain the nature of the bond formation and the cyclic voltammograms of the gold nanoparticle-modified ITO plate immersed in 0.01 M HClO₄ solutions are shown (Figure 7). The scans were initiated at -0.4 V and a reduction peak was observed at +0.69 V, which corresponds to the formation of gold oxide.

Insert Figure 7

The electrochemical evaluation of 6-MP capped gold nanoparticles is also shown in Figure 7b. Peak 1 shows the cv response for the ITO electrode (modified with gold nanoparticles) in 1 mM K₄Fe(CN)₆ at a scan rate of 10 mv/s. After the exposure of gold electrode in the 6-MP solution, a

reduction in the peak current is observed due to the binding of 6-MP with the nanoparticles and more pronounced changes in the electrochemical profile of the gold electrode occur as the 6-MP concentration increases (Figure 7b). In fact, the onset of gold oxide formation is heralded by the change to a positive potential, indicating that the 6-MP molecule has been spontaneously adsorbed on the gold surface. However at more positive potentials, a large anodic current was obtained, which should correspond to the oxidative desorption of the previously adsorbed molecule³⁵, and a reduction peak was absent when the gold electrode surface was completely adsorbed by 6-MP. The shape of the voltammograms for $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Fe}(\text{CN})_6]^{4-}$ at the coated and uncoated substrates indicates that the current is primarily controlled by linear diffusion and suggest that the monolayer does not completely block electron transfer, as these small molecules provide only a partial barrier. This decrease in both anodic and cathodic current is due to blocking behaviour of MP on gold modified electrode (Scheme 3).

Insert Scheme 3

The TEM image (Figure 8) displays clearly aggregates of gold nanoparticles: colloidal gold has an average diameter of *ca.* 18 nm. On the addition of gold, aggregation of the gold takes place (this phenomenon was previously illustrated in Schemes 2 and 3).

Insert Figure 8

Determination of the antibacterial and antifungal activity of the 6-MP-colloidal gold complex

Table 1 details the growth inhibition effected by drug-coated gold nanoparticles against both gramme positive and gramme negative organisms *viz* *Micrococcus luteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E-Coli*. It was observed that the coated colloid was most effective for gramme negative organisms and the levels of inhibition can be seen clearly in Figure 9.

Figure 9 Antibacterial activity of 6-MP and 6-MP-covered gold. (a) *Micrococcus luteus* (b) *Staphylococcus aureus* (c) *E-Coli* (d) *Staphylococcus aureus*

Table 1 Antibacterial activity of 6-mercaptopurine (6-MP)

Micro organisms	Nature of organisms	Levels of Zone of inhibition	
		Pure 6-MP	6-MP-coated gold nanoparticles
<i>Micrococcus luteus</i>	Gramme Positive	41	45
<i>Staphylococcus aureus</i>	Gramme Positive	49	57
<i>Pseudomonas aeruginosa</i>	Gramme Negative	51	63
<i>E-Coli</i>	Gramme Negative	60	72

6-MP also shows a good antifungal activity against *Aspergillus fumigatus* and *Aspergillus niger* and these activity levels were found to be enhanced on the addition of colloidal gold (Table 2 and Figure 10). The colloidal gold solution alone does not show any appreciable antifungal and antibacterial activities.

Insert Figure 10

Table 2 Antifungal activity of 6-mercaptopurine (6-MP)

Fungal Organisms	Levels of Zone of inhibition	
	Pure 6-MP	6-MP-coated gold nanoparticles
<i>Aspergillus fumigatus</i>	45	59
<i>Aspergillus niger</i>	32	37

Although these preliminary studies demonstrate the effectiveness of the gold-drug complex, the precise mechanism by which it operates is not yet known and work continues to investigate this further. Some gold-drugs, namely aurothiomalate and aurothioglucose, have already been evaluated for activity against human immunodeficiency virus (HIV) for the treatment of AIDS^{44,45} and, in the same way, gold-drugs have been explored for their effectiveness as antifungal, antibacterial and anticancer agents. Gold-drugs are used more as last-line modes of treatment for severe cases of rheumatoid arthritis in favour of organic drugs. It gives more potent and reduced toxic side effects. These properties may be due to geometry of the complex. The normal mode of

metabolite pathway and the release mechanism may be altered (favourably in some cases) by the presence of metal nanoparticles, to attain a greater efficiency and reduced side effects.

Conclusions

The binding of 6-mercaptopurine (6-MP) to colloidal gold *via* complexation through the thiol group was studied using different analytical techniques (*e.g.* U.V-Visible spectroscopy, cyclic voltammetry, transmission electron microscopy, fluorescence spectroscopy and IR spectroscopy). Fluorescence and electrochemical studies have shown the nature of interaction and will be used in future studies. The combination of gold with 6-MP results in a more potent complex compared with the individual parts.

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Figure 1 The time dependent UV-Visible obtained after mixing (0.5 mM) 6-mercaptopurine with gold nanoparticles at time intervals of 30 minutes

Figure 2 FTIR transmission spectrum of pure 6-mercaptopurine (6-MP)

Figure 3 FTIR transmission spectrum of 6-MP with gold nanoparticles (MP-AU)

Figure 4 Emission spectra of (a) free 6-MP (5 mM) and (b) the adsorbed species on gold (1 mM, 0.5 cm³) nanoparticles taken in aqueous medium at intervals of 30 min.

Figure 5 Excitation spectra taken in aqueous medium for (a) free 6-MP (5 mM) and (b) the adsorbed species on gold nanoparticles (1 mM)

Figure 6. Emission spectra taken in aqueous medium for (a) free 6-MP (5 mM) and (b-e) for the adsorbed species on gold nanoparticles. The concentration of gold solution is varied ($0.5\text{cm}^3 - 2\text{cm}^3$ of 1mM gold solution), but the analysis time is kept constant (2 minutes)

(a)

(b)

(c)

Figure 7: Cyclic voltammograms of a gold nanoparticle-modified electrode (a) in the absence of 6-MP in 0.01 M HClO₄, M, (b) in the presence of different concentrations of 6-MP in 0.01 M HClO₄. (a) 50 (b) 100 and (c) 150 μ M. The scan rate is maintained at 0.02 V s⁻¹, and (c) in 1mM K₄Fe(CN)₆ at a scan rate of 10 mV s⁻¹.

	(a)	(b)
(c)

Figure 8 Transmission electron micrographs of 6-MP-covered gold nanoparticles of average diameter 20 nm. The samples were prepared from an aqueous solution: (a) gold nano particle; (b) 6-MP-covered gold nanoparticles; (c) expanded portion of 6-MP covered gold nanoparticles

Figure 9 Antibacterial activity of 6-MP and 6-MP-covered gold. (a) *Micrococcus luteus* (b) *Staphylococcus aureus* (c) *E-Coli* (d) *Staphylococcus aureus*

Figure 10. Antifungal activities of 6-MP and 6-MP-covered gold (a) *Aspergillus fumigatus* (b) *Aspergillus niger*

Scheme1. Aggregation of 6-MP on Gold nanoparticles

Scheme 2. Synthesis of 6-mercaptopurine (6-MP)

Table 1 Antibacterial activity of 6-mercaptopurine (6-MP)

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