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International Journal of Pharmaceutics 337 (2007) 275–281
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Analytical detection and biological assay of antileukemic drug 5-fluorouracil using gold nanoparticles as probe

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Received 2 August 2006; received in revised form 13 December 2006; accepted 18 December 2006
Available online 27 December 2006

Abstract

Gold nanoparticles are reported and evaluated as probes for the detection of anticancer drug 5-fluorouracil (5FU). The nature of binding between 5FU and gold nanoparticles *via* complexation is investigated using ultraviolet visible spectrophotometry, cyclic voltammetry, transmission electron microscopy, fluorescence and Fourier transform infrared (FTIR) spectroscopy. The bound antileukemic drug is fluorescent and the quenching property of gold nanoparticles could be exploited for biological investigations. The 5FU-colloidal gold complex (Au@5FU) is observed to have appreciable antibacterial and antifungal activity against *Micrococcus luteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Aspergillus fumigatus*, and *Aspergillus niger*. The experimental studies suggest that gold nanoparticles have the potential to be used as effective carriers for anticancer drugs.

Keywords: Gold nanoparticles; 5-Fluorouracil; Microbial studies; Antibacterial and antifungal activity

1. Introduction

Materials in the nanometer size range may possess unusual and beneficial properties, which are very useful for different industrial and engineering applications including biotechnological systems. The formation of hybrid systems comprising nanoparticles and biomolecules paves the way from cell markers to biosensing (Bruchez et al., 1998; Lahav et al., 1999), bioimaging (Djalali et al., 2002) and targeted drug delivery (West and Halas, 2000). Nanoparticles have been found to be very 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 stability (Damge et al., 1997; Zeltner et al., 1991; Cappel and Kreuter, 1991). For instance, nanoparticles can be used to enhance oral delivery (Damge et al., 1997; Brannon-Peppas, 1995) 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 (Couvreur et al., 1997; Astier et al., 1998). Consequently, nanotechnology is now used as potential route to study the delivery of anti-leukaemic drugs and is also 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 animals (Beck et al., 1993; Simeonova et al., 1991; Bennis et al., 1994; Colin de Verdiere et al., 1994). Optical (Nath and Chilkoti, 2002) and electrochemical (Retna Raj et al., 2003) 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*., (Jalil and Nixon, 1990; Mu and Feng, 2003). The antibacterial property of metal nanoparticles and vancomycin capped gold nanoparticles are reported (Stoimenov et al., 2002; Gu et al., 2003).

5-Fluorouracil (5FU) occupies a special place in biomedicine, owing to its activity in cancer chemotherapy. In order to suppress the undesirable side effects associated

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doi:10.1016/j.ijpharm.2006.12.027

with anti-tumour drugs, metal-drug complexes are currently used as slow release prodrugs of 5FU. The latter is employed as coordinated components of certain transition metals like Ni, Cu, and Zn (Cho et al., 2002). 5-Flurouracil (5FU) an anticancer agent used for drug delivery has been studied in detail (Boulieu et al., 1985). A variety of uracil derivatives have been reported as antineoplastic agents. 5-Fluorouracil (5FU) is one of the standard drugs used in chemotherapeutic regimens for metastatic colorectal cancer worldwide. 5-Fluorouracil is used for the treatment of solid tumours of breast and rectum. Various analytical techniques have been employed for the detection of 5-fluorouracil. A series of uracil derivatives have been studied in detail by polarography and amperometric detection (Mircjeski et al., 2000; Bouzid and Macdonald, 1988). Recently, polymer nanoparticles has been used as carriers for the drug delivery of fluouridine, an analogue of 5FU using high performance liquid chromatography (Arbo et al., 2002). With all these potential uses of nanotechnology, there is a better way for a promising future in medicine.

In the present work, gold nanoparticles have been used as probes for the detection of the anticancer drug 5FU and evidence for their complexation and binding interaction were studied using ultraviolet UV–vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, cyclic voltammetry, transmission electron microscopy (TEM) and fluorescence spectroscopy. Furthermore, the microbial efficacy of the complex of 5FU and colloidal gold is examined with biological assays in order to evaluate its antibacterial and antifungal activities.

2. Experimental

2.1. Apparatus

Samples were characterized by UV–vis spectrophotometry (Perkin-Elmer Lambda 25). The path length was 1 cm and matched 1 cm × 1 cm cuvettes were used. TEM was undertaken employing a Tec NIE 10 instrument with an accelerating voltage of 120 kV; samples were prepared by mixing aqueous solution of 5FU (5 mM) and gold solution (1 mM). 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 were performed using CHI600B instrument in a three-electrode cell, using platinum wire as the counter electrode and Ag/AgCl (3 M NaCl) as reference electrode. 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, which was prepared in the following manner. The ITO slides were cleaned thoroughly by ultrasonication in water and washed with acetone. The cleaned glass slides were then immersed in a solution (2%, v/v) of 3-aminopropyl trimethoxysilane (APTMS) in methanol for 20 h and subsequently immersed in freshly prepared colloidal gold for another 15 h to create a submonolayer of gold nanoparticles. For electrode modification, freshly prepared gold plates were immersed (at different interval of time) in 5FU solution. The process for the electrochemical studies carried out in the present work is depicted in Scheme 1b.

2.2. Materials

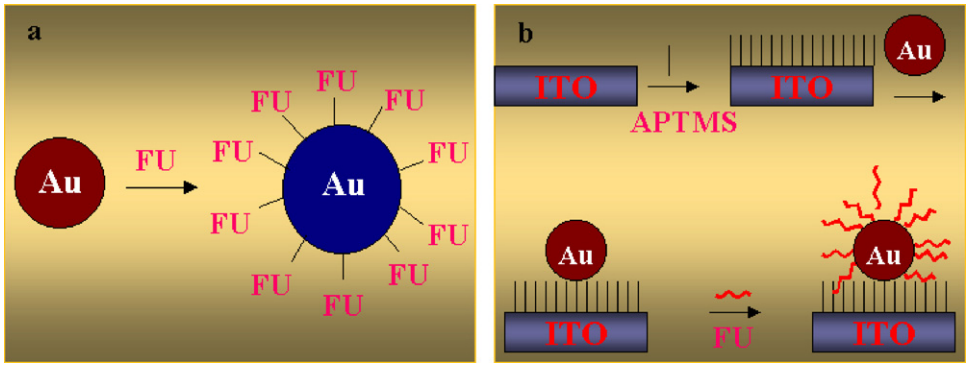
$HAuCl_4 \cdot 3H_2O$ (98%) and trisodium citrate (99%, AR) were purchased from CDH and Analytical Rasayan, respectively. 5-Fluorouracil (5FU) was purchased from Aldrich. 3-Amino propyl trimethoxy silane was obtained from Sigma–Aldrich. ITO glass plate was purchased from Asahi Beer optical, Ltd.

2.3. Preparation of citrate capped gold nanoparticles (Tom et al., 2004)

Trisodium citrate (38.8 mM, 50 cm^3) was added to a boiling $HAuCl_4$ solution (1 mM, 500 cm^3). After the addition, the previously yellow colored solution of gold chloride turned wine red in color and gave a characteristic absorbance at 518 nm in the UV–vis spectrum. From the TEM measurements, the size distributions of the particles were found in the range of 17–18 nm.

2.4. Preparation of 5FU coated gold nanoparticles

For the preparation of 5FU capped gold nanoparticles, 1 mM (50 cm^3) of the synthesized gold nanoparticles were mixed with 5FU (5 mM) in H_2O (25 cm^3) and stirred effectively for 12 h



Scheme 1. (a) A schematic diagram elucidating the aggregation of 5FU on gold nanoparticles and (b) a schematic representation depicting the SAM formation of 5FU on gold nanoparticles for electrochemical studies.

until the wine red colour became blue. The 5FU coated gold nanoparticles were used for further studies.

2.5. Details of microbial assay

Antibacterial and antifungal activities were studied using a disk diffusion method, wherein a suspension of Gram-positive, Gram-negative organisms and a few fungal 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, 5FU and Au@5FU were placed on agar plates and the plates were left for 1 h 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 h, and observed for antibacterial and antifungal activities by determining the diameters of the zones of inhibition for each of the samples.

3. Results and discussion

3.1. Optical properties of drug coated gold nanoparticles

The plasmon band observed for the wine red colloidal gold at 518 nm in the UV–vis spectrum is characteristic of gold nanoparticles. Pure drug shows a maximum at 271 nm which arises due to n–π* transition of 5-fluorouracil molecule and, with the addition of colloidal gold to 5FU, both the bands at 271 and 518 nm pertaining to pure drug and Au colloids decreases in intensity. This decrease is accompanied by emergence of an additional peak at 650 nm (Fig. 1). The appearance of this new peak is a consequence of aggregation, which is due to interparticle interaction between the adjacent gold nanoparticles upon the addition of drugs. The latter can be verified by a color change from wine red to blue with the addition of drug to colloidal gold. The appearance of a new peak is due to the aggregation of gold nanoparticles caused by the replacement of citrate molecules with FU leading to the formation of gold-drug complex (Scheme 1a). This can be attributed to the greater electrostatic attraction of active groups in FU with gold nanoparticles than citrate groups, which is responsible for the additional band at higher wavelengths. It

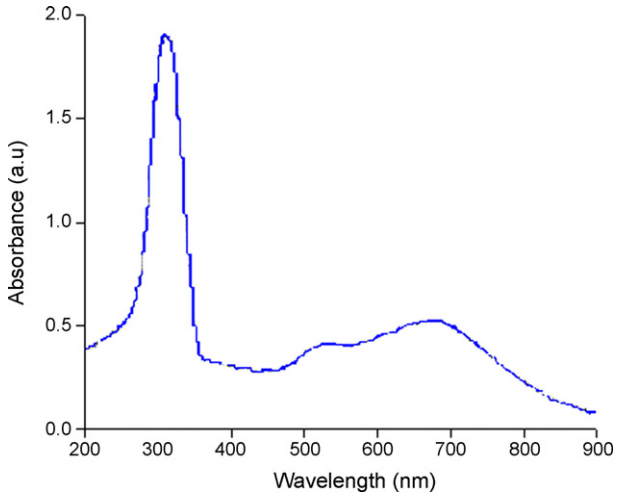


Fig. 1. The UV–vis spectrum obtained after mixing 0.5 mM 5FU solution with gold nanoparticles (after 6 h).

has been shown theoretically and experimentally that aggregation of gold nanoparticles leads to another plasmon absorption at longer wavelengths when the individual nanoparticles are electronically coupled to each other (Tom et al., 2004; Kreibig et al., 1981). The oscillating electrons in one particle feel the electric field due to the oscillation of the free electrons in a second particle that can lead to a collective plasmonic oscillation of the aggregated system. Citrate ions are readily replaced by a –NH ligand on gold nanoparticle surfaces. This ligand exchange reaction provides an important means for the chemical functionalization of the nanoparticles and greatly extends the versatility of these systems.

3.2. FT-IR studies

The binding interaction of drug with Au(0) was further investigated using FT-IR studies. The IR spectra of free drug and Au@5FU are shown in Fig. 2. Pure drug shows band at 3450 cm^{-1} corresponding to the stretching frequency of –NH group. Other absorption bands at 1684 and 1420 cm^{-1} are due to C=C and C–N stretching vibrations, respectively (Fig. 2a).

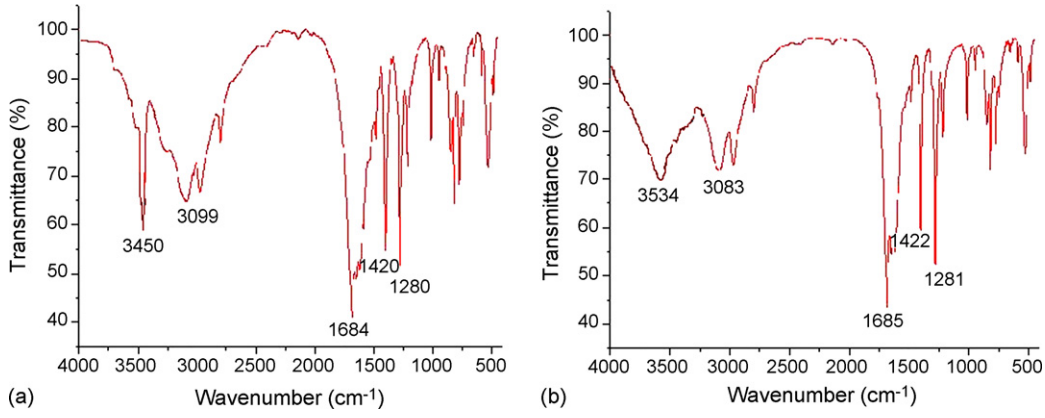


Fig. 2. FT-IR transmission spectrum of (a) pure 5FU; (b) 5FU with gold nanoparticles (Au@5FU).

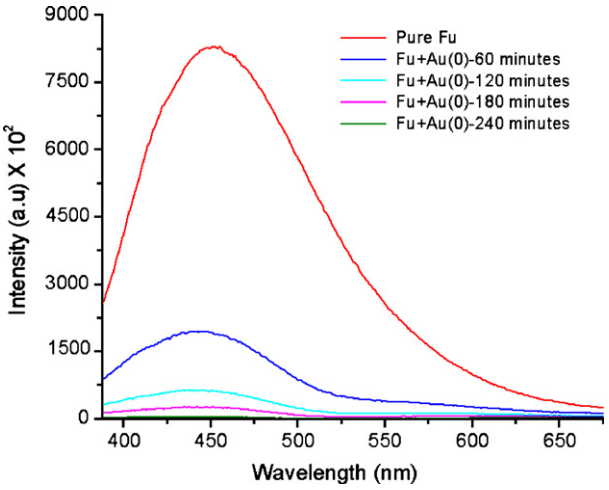


Fig. 3. Emission spectra recorded for free 5FU (5 mM) and 5FU with Au(0) at various time intervals.

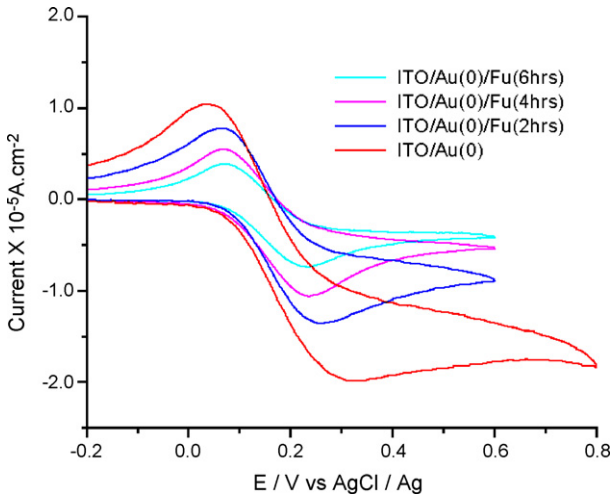


Fig. 4. Cyclic voltammograms of gold nanoparticles modified ITO electrodes [ITO/Au(0)] and 5FU coated gold [ITO/Au(0)/5FU] electrodes in 1 mM $K_4Fe(CN)_6$ at a scan rate of 10 mV s^{-1} using Ag/AgCl as reference and pt wire as counter electrode.

In the case of drug coated gold colloids (Fig. 2b), there are no changes in the absorption frequencies of all other groups except NH which is now broadened and shifted to higher wavelengths at 3534 cm^{-1}. From this it could be understood that it is the free –NH group that is likely to be involved in binding on the gold nanoparticle surface. It is well known that gold has a strong affinity towards amino groups (Aslam et al., 2004).

3.3. Binding interaction from fluorescence studies

Fluorescence studies offer an excellent probe for confirming the binding of drug with gold nanoparticles as it has been previously reported that gold metal efficiently quenches the emission of many fluorophores (Pagnot et al., 1999; Kamat et al., 2002; Dulkeith et al., 2002). Huang and Murray have described the quenching of small dyes molecule by gold nanoparticles (Huang and Murray, 2002) while Dubertret and group have used gold nanoparticles as an effective proximal quencher in DNA molecular beacons (Dubertret et al., 2001). In the present investigation, 5FU was highly fluorescent but with the addition of gold colloids, quenching of fluorescence was observed. Gold nanoparticles are non-fluorescent while pure 5FU showed a high and a broad emission centered at around 436 nm when excited at a wavelength of 360 nm. In the presence of gold nanoparticles, the fluorescence intensity was reduced and this quenching of intensity can be attributed to the electronic interactions between the drug molecule and gold nanoparticles. The emission spectra was recorded at every 1 h for a period of 4 h (Fig. 3). Due to the binding of –NH group on Au nanoparticle surfaces, the electronic environment is altered which results in quenching of fluorescence. It can be seen from the figure that with an increase in reaction time, the fluorescence intensity is drastically quenched. With an increase in reaction time, more amount of free 5FU gets bound to gold nanoparticles and hence this causes a further decrease in fluorescence intensity. From these studies, it could be concluded that 5FU gets bound to gold nanoparticles probably through an electrostatic interaction between the drug and gold moieties.

3.4. Electrochemical evaluation of drug coated gold colloids

To further support the binding of 5FU moiety on gold nanoparticles, cyclic voltammetry was employed wherein, Au(0) modified ITO was used as working electrode. Electrochemical reactions involving the redox reaction of an aqueous solution of 1 mM $[Fe(CN)_6]^{3-/4-}$ with 1 M KCl was employed to account for the electron transfer between the electrode and Au(0) and its effects with the addition of 5-fluorouracil. The redox reaction of $[Fe(CN)_6]^{3-/4-}$ using 5FU coated gold electrodes were investigated and the electrochemical evaluation is shown in Fig. 4. The first CV shows the response for the ITO electrode modified with gold nanoparticles in 1 mM $[K_4Fe(CN)_6]$ at a scan rate of 10 mV/s. The modified electrode exhibits fairly well defined oxidation and reduction peaks and, after the exposure of the gold electrode with 5FU, a decrease in peak current was observed. The CV was recorded at different time of exposure of Au electrodes in 5FU. It was observed that with an increase in exposure time, the redox currents are decreased further. Thus more and more amount of 5FU gets adsorbed on Au surfaces with an increase in the exposure time of Au electrodes to 5FU. The decrease in redox currents with the exposure of Au electrodes in 5FU indicates that 5FU molecules have covered a large area of Au surface and blocks the interaction between Au and Fe ions thus producing a decreased current. Although a decreased current is observed, a complete reduction of the redox currents was not observed which indicates that 5FU monolayer does not completely block the electron transfer between Au(0) and Fe ions. There must be some sort of defects or voids on the 5FU coated Au electrodes, which allows the redox reaction to take place between Au and Fe ions. The shape of the voltammograms for $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ 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

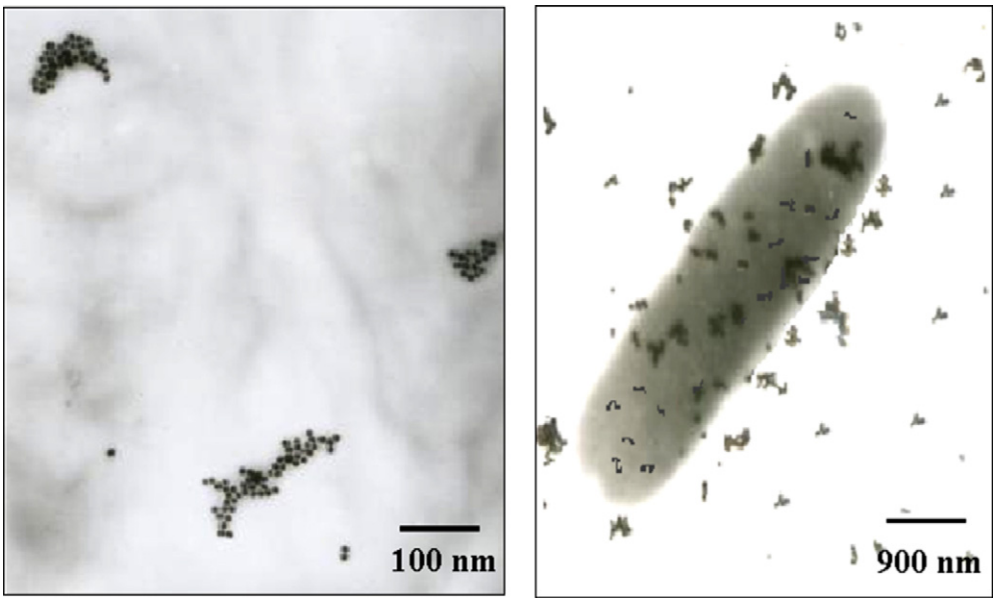


Fig. 5. (a) Transmission electron micrographic images of 5FU-coated gold nanoparticles. (b) TEM images of *E. coli* after being treated with 5FU depicting the penetration of nanoparticles into the *E. coli*.

only a partial barrier. Thus 5FU binds on Au surface affecting its electrochemical properties. From these electrochemical studies, it is clear that 5FU molecules have a strong propensity to bind on Au nanoparticle surface. This decrease in both anodic and cathodic current is due to blocking behavior of 5FU on gold modified electrode (Scheme 1b). It is well known that the cathodic (reduction) and anodic (oxidation) faradaic current peaks at the metal are completely suppressed in the presence of a defect-free SAM (Wang et al., 2000).

3.5. TEM Images of drug coated gold nanoparticles

The TEM image (Fig. 5a) clearly displays aggregates of gold nanoparticles: colloidal gold has an average diameter of *ca.* 18 nm. With the addition of 5FU, aggregation of the gold takes place and this phenomenon is illustrated in Scheme 1a.

3.6. Determination of antibacterial and antifungal efficacies of 5FU-colloidal gold complex (Au@5FU)

The binding of the anticancer drug 5FU with gold nanoparticles has been established using various analytical techniques in the previous sections. Using this property, we were further interested in extending our work towards biological investigations in order to establish that Au(0) nanoparticles could act as an effective drug carrier in cancer therapy. Hence, an investigation was undertaken to study and compare the microbial efficacies of 5FU coated gold colloids with various strains of Gram-positive and Gram-negative bacteria like *Micrococcus luteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and a few fungal organisms such as *Aspergillus fumigatus* and *Aspergillus niger* have been selected to probe the antibacterial and antifungal efficacies. Some of the previous studies have utilized Au(0) for investigating the antibacterial effectiveness of antibiotics protected Au(0) against *E. coli* (Stoimenov et al., 2002; Gu et al., 2003). To the best of our knowledge, there are no reports detailing the microbial properties of anticancer drugs with gold nanoparticles. Also the antifungal properties of gold nanoparticles are the first to be reported in our present investigation.

Table 1 details the growth inhibition effected by drug-coated gold nanoparticles against both Gram-positive and Gram-negative organisms *viz. M. luteus*, *S. aureus*, *P. aeruginosa* and *E. coli*. The observed difference in activity with various antifungal and antibacterial might be attributed to the small particle size of Au(0), large surface area and high penetrating power and hence could effectively bind to the substrates on the outer membrane and cell membranes of organisms. Moreover, nanogold possesses well-developed surface chemistry, chemical stability and appropriate smaller size (18 nm in diameter and 250 times smaller than a bacterium), which makes easier to penetrate the microorganism cell walls. Nanoparticles are also able to maintain a constant shape and size in solution. Despite the fact that the mechanism of the interaction between nanoparticles

Table 1
Antibacterial activity and Antifungal activity of 5FU coated (1.0 μg/mL) gold colloids with their respective level of zone of inhibition

Micro organisms	Nature of organisms	Levels of zone of inhibition	
		Pure 5FU	Au@5FU
Bacterial organisms			
M. luteus	Gram-positive	19	27
S. aureus	Gram-positive	20	31
P. aeruginosa	Gram-negative	23	35
E. coli	Gram-negative	27	43
Fungal organisms			
A. fumigatus		23	32
A. niger		19	29

and the constituents of the outer membrane of microorganisms are still unanswered, it might be that the particles interact with the building elements of the outer membrane causing structural changes, degradation and finally cell death. Comparatively, the drug-coated colloids were most effective against Gram-negative organisms. This may be explained based on the nature of the material present in cell wall. Gram-positive organisms generally have thick mesh like cell wall made of peptidoglycan layer, whereas Gram-negative organisms possess a thin cell wall with peptidoglycans. Thus an easier permeability could be achieved in the case of Gram-negative organisms. This fact is further supported by the TEM images (Fig. 5b), which confirm the approach and penetration of nanoparticles into the *E. coli*, which, in turn, support the enhancement of antibacterial activity.

5FU–Au complex also shows a good antifungal activity against *A. fumigatus* and *A. niger* (Table 1). 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 their activity against human immunodeficiency virus (HIV) for the treatment of AIDS (Okada et al., 1993; Yamaguchi et al., 2001) and, in the same way, gold-drugs have been explored for their effectiveness as antifungal, antibacterial and anticancer applications. Gold-drugs are used more as last-line modes of treatment for severe cases of rheumatoid arthritis in favor 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 (favorably in some cases) by the presence of metal nanoparticles, to attain a greater efficiency and reduced side effects.

4. Conclusion

The binding of 5-fluorouracil (5FU) to colloidal gold *via* complexation through the –NH group was studied using different analytical techniques (e.g. UV–vis spectroscopy, FT-IR, cyclic voltammetry, transmission electron microscopy, fluorescence). The nature of interaction as evidenced from fluorescence and electrochemical studies will have profound applications in biological sciences in the future studies. Further, from the data resulted from present study, it is inferred that the combination of anticancer drug with gold nanoparticles have appreciably higher activity against Gram-negative bacteria. The combination of gold with 5FU results in a more potent complex compared with the individual parts and this property would be further exploited in living systems.

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