

The role of nanogold in human tropical diseases: research, detection and therapy

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Published online: 6 April 2013

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Introduction

The Special Programme for Research and Training in Tropical Diseases, based at and executed by the World Health Organization (WHO), states that these maladies affect millions of people across the world [1–5]. They are more common in tropical and subtropical regions, as in temperate climates the cold season is able to control the insects population, which are the most common disease carriers or vectors. The flies and mosquitos may carry parasites, bacteria or viruses that can infect humans and animals through a “bite”, which transmits the illness agent through a subcutaneous blood exchange. Some factors like deforestation, exploration of tropical areas, and increasing international air travel to tropical regions lead to an increase of tropical diseases. For some of them, unfortunately, vaccines are still not available.

The recent explosive growth of nanogold applications in many areas [6–9], also comprises contributions to the health field [10–14]. In particular, gold can have a direct role in treatment and detection of tropical diseases, as shown by some studies found in literature. Besides some examples of molecular gold used directly against parasites and in research dealing with animal parasitic diseases [15–17], gold nanoparticles and gold complexes can also play a significant role that goes beyond the classical approach of confirmation diagnosis,

based on detection of parasites in either blood or lymph by microscopy, as it will be explained below.

Trypanosomiasis

About the diseases

There are two different types of human trypanosomiasis [1, 2]: the human African trypanosomiasis and the American trypanosomiasis. The latter is more studied, but it is important to also refer the progresses made so far on the former.

The human African trypanosomiasis, also known as “sleeping sickness”, is a well-known tropical disease, caused by the protozoan parasites *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense*. It is spread by a bite of an infected tsetse fly, which erupts into a red sore. Within a few weeks, it causes fever, swollen lymph glands, aching muscles and joints, headaches and irritability. In advanced stages, the disease attacks the central nervous system, causing changes in personality, alteration of the circadian rhythm, confusion, slurred speech, seizures, and difficulty walking or talking. These problems can develop over many years in the *gambiense* form and some months in the *rhodesiense* form. If not treated, it can cause death. An early diagnosis is of extreme importance for a better control of this sickness.

The American trypanosomiasis, or Chagas disease, is caused by the protozoan parasite *Trypanosoma cruzi*, generically schematized in Fig. 1. It is found mainly in Latin America, where it is mostly transmitted to humans by the faeces of triatomine bugs [2]. This disease can cause death from myocarditis or meningoencephalitis during its acute phase, in less than 5–10 % of symptomatic cases. The evolution for the chronic phase (10–30 years after infection) can result in cardiac, digestive

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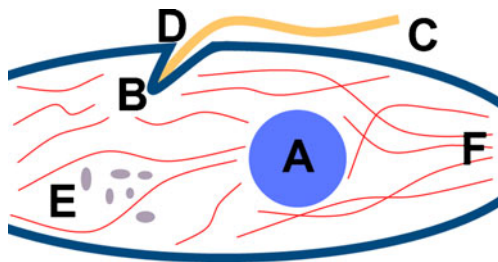


Fig. 1 *Trypanosoma cruzi* cell schematic representation (adapted from [87, 88]). **a** Nucleus, **b** flagellar pocket, **c** flagellum, **d** cytostome, **e** cytoplasmic vesicles, **f** cytoskeleton

(megaesophagus and megacolon), or cardiodigestive problems in 30–40 % of the infected patients [18].

Microscopy assays using nanogold

In 1997, Magez et al. [19], knowing that the protozoan *Trypanosoma brucei* is lysed by the cytokine tumor necrosis factor- α (TNF- α), prepared TNF- α gold nanoparticles (TNF- α -AuNP) using commercial 10 nm gold beads. These TNF- α -AuNP were endocytosed via coated pits and vesicles and are directed towards lysosome-like digestive organelles. The localization of TNF- α binding sites on intact parasites and a better understanding of intracellular uptake of TNF- α were possible precisely with the help of the conjugated colloidal gold particles. The bulk of the TNF- α gold labeling was localized in the flagellar pocket, where beads concentrated in coated pits. Sporadically, TNF- α -AuNP were found in association with the flagellum in the flagellar adhesion zone, at the entrance of the flagellar pocket, or in association with tiny filamentous material at more distant regions of the flagellum.

Soeiro et al. [20] performed ultrastructural analysis of the endocytic process (cellular uptake) using horseradish peroxidase (HRP) enzyme coupled to colloidal gold particles (HRP-AuNP). The "in vitro" findings suggest that cardiomyocytes (cells of cardiac muscle) mannose receptors, localized at the sarcolemma (muscle cell membrane), mediate *T. cruzi* recognition and can be down-modulated by parasite infection.

Okuda and co-workers [21] used ultrathin sections of *T. cruzi* epimastigote forms. These assays, which reveal gold particles at the opening of flagellar pocket, concentrated in the cytostome region, were possible through immunogold labeling (a staining technique used in electron microscopy, where colloidal gold nanoparticles are usually attached to secondary antibodies, which are attached to primary antibodies designed to bind a specific protein or other cell component). The protocol consists in cytoskeletons incubation in the first antibody, which recognizes the cytoskeletons elements; then incubated in the second antibody, which recognizes the first antibody and have attached a 15 nm gold particle. Using electron microscopy to visualize the gold nanoparticles

(AuNP), and consequently the known proximity of the elements recognized by the antibody–antibody–AuNP configuration, the relationship between the cytostome, an endocytic organelle, and the flagellum was described for the first time. Gold has high electron density which increases electron scatter enabling high contrast images.

A similar protocol allowed Monteiro et al. [22], in 2001, to describe the localization of chagasin, an endogenous tight-binding cysteine protease inhibitor in *T. cruzi*. Gold-labeled antibodies localized chagasin to the flagellar pocket and cytoplasmic vesicles of trypomastigotes and to the cell surface of amastigotes (the former are found in human blood and the later in tissues). Thin sections were incubated with affinity purified rabbit anti-chagasin followed by gold-conjugated goat anti-rabbit Immunoglobulin G (IgG).

Silva et al. [23] evaluated the presence and distribution of the Ssp4 antigen in the different amastigote *T. cruzi* populations using gold-labeled antibodies, allowing the observation with transmission electron microscopy. Goat anti-mouse IgG was labeled with 10 nm gold particles. These were mainly located inside cytoplasmic vesicles and the flagellar pocket, suggesting that Ssp4 is released by exocytosis into the flagellar pocket.

Recently, Acosta et al. [24] used immunogold electron microscopy analysis for providing a new insight to better understand the molecular pathogenesis of Chagas heart disease. They showed that there is a common epitope between cruzipain (a lysosomal major antigen from *T. cruzi*) and either myosin or other cardiac *O*-linked *N*-acetylglucosamine containing proteins.

Eger and Soares [25] described the visualization by confocal microscopy of ingested gold (15 nm) labeled transferrin in epimastigote forms of the protozoan *T. cruzi*. This is a promising imaging tool to explore the endocytic pathway in trypanosomes (and eventually adapted for other protozoans).

Detection methods using gold

In 2003, Diniz et al. [26], prepared a gold electrode for adsorbing a polypeptide chain formed by recombinant antigens: cytoplasmic repetitive antigen (CRA) and flagellar repetitive antigen (FRA) of *T. cruzi*. The goal was to develop a biosensor for Chagas disease, based on impedance spectroscopy, with the behavior of CRA/FRA antigens adsorbed on gold and platinum electrodes being investigated. The team also used platinum electrodes, which yielded similar results as those presented by gold; however, the marked influence of oxygen dissolved in solution on the electrochemical response of platinum, requires extensive deoxygenation of solutions prior each experiment. Consequently, gold electrodes have advantages. Under proper conditions, it is possible to distinguish between serum positive and negative to Chagas disease.

Joining the last two methods (as an “immunotechnique” is applied, but gold acts as a supporting electrode for sensing purposes, instead of labeling), Ferreira et al. [27] immobilized Chagas disease antigens in a gold surface, and the anti-*T. cruzi* antibodies present in the serum sample were captured by the antigen, remaining connected to the solid phase. The human anti-IgG antibody conjugated to peroxidase (HRP) then reacted with immunocaptured anti-*T. cruzi* antibodies, if any present. The detection and quantification was ensured by peroxidase activity in presence of H_2O_2 and, in the presence of the iodide, this ion reacted with the reduced peroxidase. Under these conditions, the current intensity of I_2 reduction was proportional to the amount of anti-*T. cruzi* antibodies in the serum real sample. This assembling is schematized in Fig. 2.

In a very similar approach, Ribone and co-workers [28] prepared bioelectrodes to detect IgG antibodies occurring in sera of patients suffering from American trypanosomiasis. The main difference from the previous description [27], is the use of two different thiols, 3-mercaptopropionic acid (MPA), 3-mercaptopropanesulfonic acid (MPSA), which provide sulfonate or carboxylic residues, for the electrostatic binding of the antigen. Figure 2 also applies to the assembling described in this work, where the linkage to the gold electrode is described generically as “thiol”.

Foguel et al. [29] used the same principle for the construction of an amperometric immunosensor. The novelty here was the gold based electrode being obtained from a recordable compact disc (CD-R), then modified with 4-(methylmercapto)benzaldehyde for the immobilization of Tc85 protein of the *T. cruzi*. To access the metal layer of the CD-R, concentrated HNO_3 was added on the surface and after 5–10 min the protective layers were totally removed. It is well known that organic monolayers with thiol or disulfide groups on the electrode surface are of great

interest due to the sulfur binding strongly to the gold surface. A molecule that presents this property is 4-(methylmercapto)benzaldehyde (SBZA), which has a mercapto group and maintains a free aldehydic group. In order to link the CD-R fragment and allow the amperometric process, a laminated copper wire was fixed and insulated with polytetrafluoroethylene (PTFE), so that the wire does not come into contact with the electrolyte solution, thus avoiding the oxidation and reduction of this copper.

Using a similar methodology, a microfluidic system coupled to a screen-printed carbon electrode (SPCE) was developed by Pereira and co-workers [30] for the quantitative determination of IgG specific antibodies present in serum samples of patients with Chagas disease. This time, HRP in the presence of H_2O_2 catalysed the oxidation of 4-*tert*-butylcatechol (4-TBC) and its back electrochemical reduction was detected on a modified electrode. The authors claimed that, compared with traditional IgG detection techniques, the immunosensor based on microfluidic technology showed a decrease in sample and reagent consumption, faster response times for analysis, and good reproducibility and did not require highly skilled technicians or expensive and dedicated equipment.

Deborggraeve et al. [31] developed a simple and rapid test for detection of amplified *T. brucei* DNA, the human African trypanosomiasis–polymerase chain reaction–oligochromatography (HAT–PCR–OC). In this case, gold was used essentially as a colorimetric indicator. PCR products were visualized on a dipstick through hybridization with a gold-conjugated probe (oligochromatography), occurring in 5 min. The lower detection limit of the test was 5 fg of pure *T. brucei* DNA; one parasite in 180 μ l of blood is still detectable. The authors concluded that HAT–PCR–OC is a promising new tool for diagnosis of “sleeping sickness” in laboratory settings.

Nanogold chemotherapy

Nyarko et al. [32] described the effects of aqueous Au(III) and his metalloporphyrin derivative on *T. brucei brucei* growth in culture. While Au(III) porphyrin was effective against the parasites at concentrations above 4.8×10^{-6} M, aqueous Au(III) ion was toxic to the trypanosomes at concentrations as low as 2.0×10^{-7} M, due to free radicals formation. Although the parasite tested is responsible for an animal African trypanosomiasis (and this review deals mainly with human tropical diseases), the work of Nyarko et al. shows that gold might have a promising role in the battle against these noxious organisms.

A series of seven papers regarding metal-based chemotherapy against tropical diseases was published by Sánchez-Delgado and co-workers [33–39], from 1993 to 2004. Navarro, who wrote a very interesting review in 2009 about

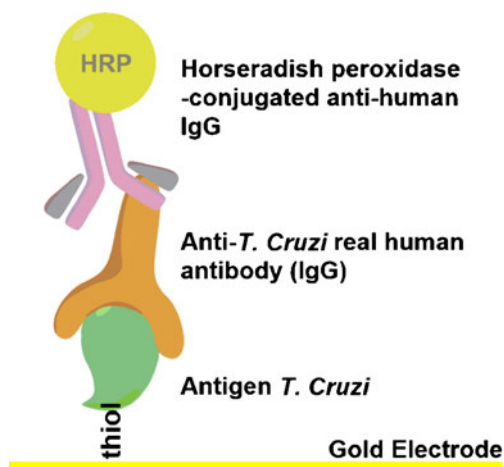


Fig. 2 Immunosensors supported on a gold electrode (adapted from [29, 30])

gold complexes as potential anti-parasitic agents [40], published since 1997, together with different co-workers, also some papers in this series [35, 36, 38, 39]. In one of those publications [36], the authors dealt with a group of metal–clotrimazole complexes evaluating their activity against *T. cruzi*, including $\text{AuCl}_3(\text{clotrimazole})$. The clotrimazole (CTZ) is an azole derivative, and this family of compounds has been developed as chemotherapeutic agents for the treatment of fungal diseases on the basis of their properties as sterol biosynthesis inhibitors, which can also affect some parasites [36]. This $\text{AuCl}_3(\text{CTZ})$ complex (Fig. 3, middle) showed an anti-*T. cruzi* epomastigotes activity only slightly higher than the free CTZ (60 % vs. 58 %), with a poor performance compared other metal–CTZ complexes [36, 40]. In another paper [38], Navarro et al. considered other azol derivative (ketoconazole [KTZ]) and other Au precursor, preparing $[\text{Au}(\text{CTZ})(\text{PPh}_3)]\text{PF}_6$ (Fig. 3, top) and $[\text{Au}(\text{KTZ})(\text{PPh}_3)]\text{PF}_6 \cdot 2\text{H}_2\text{O}$ (Fig. 3, bottom) complexes. Both complexes (1 μM) showed an inhibition of the proliferation of the *T. cruzi* epimastigotes around 70 %, while the free CTZ has no effect on the growth and KTZ only inhibited 39 % at the same concentration [38, 40].

It is also important to mention the role of gold in biolistic (or biological ballistics, consisting in transfecting cells by bombarding them with microprojectiles coated with DNA) immunization using a stable DNA–gold precipitate [41]. As the target population of a *T. cruzi* vaccine lives predominately in poorer rural areas in South America, the use of such DNA–gold precipitate, which does not require a cold chain, is an attractive method for vaccination.

Leishmaniasis

About the disease

Some *Leishmania* parasitic protozoa are the cause of leishmaniasis in humans. The disease is spread through the bite of phlebotomine sandflies, which breed in forest areas, caves and adobe brick houses, where most of the transmission to humans takes place. This disease can be divided in four main types [3]:

- (1) Cutaneous forms, with skin ulcers usually on exposed areas, such as the face, arms and legs, that usually heal within a few months, but leaving scars.
- (2) Diffuse cutaneous leishmaniasis, which produce disseminated and chronic skin lesions and it is difficult to treat.
- (3) Mucocutaneous forms, where the lesions can destroy the mucous membranes of the nose, mouth, throat cavities and surrounding tissues.
- (4) Visceral leishmaniasis (VL), which is characterized by high fever, considerable weight loss, anemia,

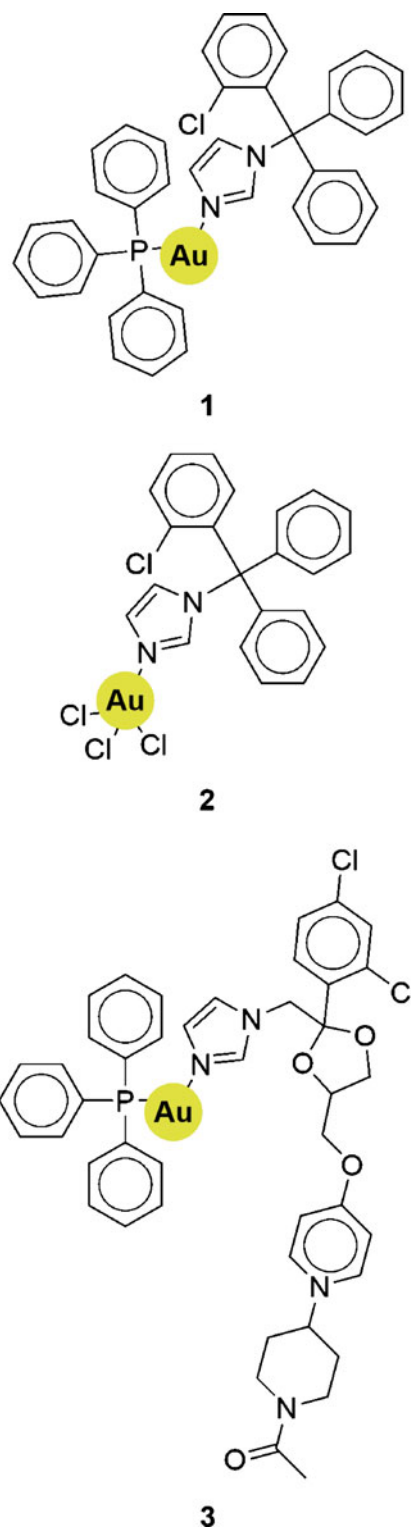


Fig. 3 Au^{I} -clotrimazole (1), Au^{III} -clotrimazole (2) and Au^{I} -ketoconazole (3) complexes (adapted from [40], counterions omitted for simplification)

spleen and liver swelling. If not treated, the disease can have a fatality rate, as high as 100 %, within 2 years.

Microscopy assays using nanogold

Santos et al. [42], in 1991, used transmission electron microscopy for observation of lectins associated with colloidal gold particles (15 nm). AuNP bound to concanavalin A (a lectin protein) were found randomly and sparsely distributed on the *Leishmania donovani chagasi* surface, along the cell body and along the flagellum. The pre-treatment of promastigotes with trypsin did not interfere with the binding of lectins to the parasite or alter the parasite ultrastructure, showing a good resistance to this protease. However, a pre-treatment with 2-mercaptoethanol increased the colloidal gold density, showing that this last compound exposed second-order concanavalin A receptors.

The intracellular fate of human transferrin (HTf) in macrophages infected by *Leishmania* was investigated by Borges et al. [43], i.e., binding the HTf to gold nanoparticles complexes for further observation in order to understand the location and processing of HTf across time. Within parasites, HTf was found in cysteine-proteinase-rich structures, suggesting that the protein can be endocytosed by intracellular amastigotes and sorted to the parasite endosomal–lysosomal compartments rather than being recycled.

Sengupta et al. [44] explored the relation between hemoglobin endocytosis and the parasite, using immunogold techniques, contributing to understand the hemoglobin destruction, a critical aspect in the disease. A specific high affinity binding site on the surface of *Leishmania donovani* promastigotes mediated rapid internalization and degradation of hemoglobin. When *Leishmania*, previously incubated with hemoglobin at 4 °C, were treated with a rabbit anti-hemoglobin antibody, followed by an anti-rabbit IgG conjugated with colloidal gold, accumulation of gold particles could be seen in the flagellar pocket. After incubation with hemoglobin–gold conjugates at 25 °C or 37 °C, the particles accumulated in discrete intracellular vesicles, suggesting the internalization of hemoglobin at both temperatures. Quantitation could be carried out by counting the number of gold particles internalized by *Leishmania* for each set of experimental conditions.

Detection methods using nanogold

Zijlstra et al. [45] evaluated a strip test employing recombinant K39 (rK39) antigen and protein-A (a surface protein originally found in the cell wall of the bacterium *Staphylococcus aureus*)/colloidal gold as read-out agents. They stated that this test has the ideal format for use in the field but also has important limitations and should be used with caution. In general, a positive test result in a patient who presents with

the classical clinical features of VL supports the diagnosis. It should be noted that a positive test result may be the result of previous (subclinical) infection and therefore not relevant to the current illness. Under field conditions, it is not always clear from the history whether a patient who reports previous treatment for VL was correctly diagnosed at the time. Such test was used recently by Carreira et al. [46] in order to study the natural infection with *Leishmania infantum* on opossums (*Didelphis aurita*), considered natural hosts of parasites, suggesting their important role in the epidemiology of VL. This work allowed the authors to present the first report of amastigotes in the tissues of *Didelphis aurita* naturally infected with *L. infantum*.

Ramos-Jesus and co-workers [47] prepared a quartz crystal gold electrode where the recombinant antigen of *Leishmania chagasi* rLci2B-NH6 was tightly immobilized on a quartz crystal gold electrode by self-assembled monolayer based on short-chain length thiol. This device was planned for the canine visceral form; however this assembling seems to be promising also for human leishmaniasis. It is important to distinguish from the similar examples given before [27, 28], as no immunotechnique is applied to sensor construction in this case. The amine groups of cysteamine provided reaction sites for covalently bind to the glutaraldehyde. The rLci2B-NH6 antigen was then immobilized through a Schiff base via glutaraldehyde by a histidine tail (Fig. 4). The Schiff base allows major exposure of epitopes and a reduced steric hindrance. The response was obtained by recognition of immobilized rLci2B-NH6 antigen.

Moreno et al. [48] designed a novel SELEX procedure (a methodology in which single stranded oligonucleotides are selected from a wide variety of sequences based on their interaction with a target molecule) using colloidal gold to select high affinity single stranded DNA aptamers that bind specifically to *L. infantum* KMP-11. Kinetoplastid membrane protein-11 (KMP-11) is a major component of the cell

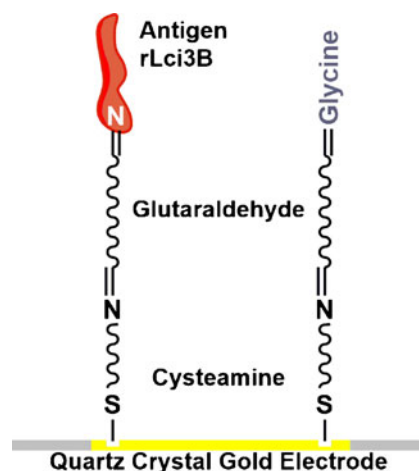


Fig. 4 Quartz crystal gold electrode supporting rLci3B-NH6 via cysteamine and glutaraldehyde (adapted from [47]). Glycine presence represents the blocking treatment in order to minimize the nonspecific binding

membrane of kinetoplastid parasites. Although its function is not known, the fact that KMP-11 is a cytoskeleton-associated protein suggests that it may be involved in mobility or in some other aspects of the flagellar structure. The method is based on the binding of the target protein to colloidal gold in order to achieve a higher amount of that protein for further purification by centrifugation. This novel methodology is very easy to use and cheaper than others that are currently being used. The same authors also developed an electrochemical biosensor based on aptamers that can recognize and specifically report the presence of *L. infantum* KMP-11. The target protein was conjugated with gold nanoparticles and the complexes electrodeposited on gold screen-printed electrodes (SPE) [49]. Using this method, Moreno et al. were able to detect 25 mg/ml of KMP-11.

Nanogold chemotherapy and thermotherapy

Recently, Ilari et al. [50] reported on the interesting antiparasitic actions of a well-known antiarthritic agent, the gold(I)-containing drug Auranofin. As trypanothione reductase, a key enzyme of *L. infantum*, contains a dithiol motif at its active site and gold(I) compounds are known to be highly thiophilic, it was found that Auranofin behaved as an effective enzyme inhibitor. It might also be a potential antileishmanial agent, as it was also found that Auranofin is able to kill the promastigote stage of *L. infantum* at micromolar concentration. These important findings will certainly contribute to the design of new drugs against this disease.

Navarro et al. [51] studied a gold complex prepared from a dypirido[3,2-*a*: 2,3-*c*]phenazine ligand, $[\text{Au}(\text{dppz})_2]\text{Cl}_3$ (Fig. 5), that interacts with DNA by intercalation mode. The large leishmanicidal activity of this complex was associated to the cellular processes involving parasite DNA, constituting a new promising chemotherapeutic alternative in the search for the cure of leishmaniasis [51].

More recently, Barboza-Filho et al. [52] studied the growth of *Leishmania brasiliensis* promastigotes in culture, using natural rubber membranes with and without gold nanoparticles. It was observed that the increase of AuNP caused a decrease in the number of promastigotes

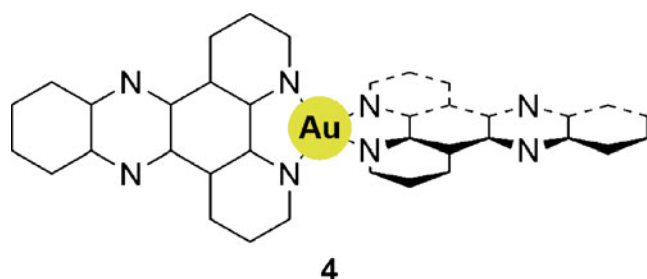


Fig. 5 Complex prepared by Navarro et al. from dypirido[3,2-*a*: 2,3-*c*]phenazine ligands (perpendicular to each other) (adapted from [51])

in culture medium. These results are advanced as a possible solution for developing a flexible “band-aid” for skin lesions, inhibiting the population growth of parasites in the lesions.

A recent study from Sazgarnia et al. [53] determined the efficacy of thermotherapy in the presence of AuNP and microwave radiation (2,450 MHz) on the survival of *Leishmania major* promastigotes and amastigotes. It was shown that the presence of AuNP during microwave irradiation (after cell incubation) was more lethal for promastigotes and amastigotes when compared to microwave radiation alone. This shows that AuNP are a promising new approach to treat leishmaniasis in the future.

Malaria

About the disease

Malaria is caused by the *Plasmodium* parasite, which is transmitted through the bites of infected mosquitos. After entering in the human body, the parasites multiply in the liver and infect the red blood cells. Fever, headache, and vomiting are symptoms of malaria and usually appear between 10 and 15 days after the mosquito bite. Malaria can quickly become life-threatening by disrupting the blood supply to vital organs and, in many parts of the world, the parasites have developed resistance to a number of known medicines. About 3.3 billion people (almost half of the world's population) are at risk of getting malaria. Every year, this leads to about 250 million cases and nearly 1 million deaths. Consequently, a prompt and effective treatment is crucial [4].

Microscopy assays using nanogold

In the study performed by Bhowmick et al. [54], immunogold electron microscopy was used for sub-cellular localization of *Plasmodium falciparum* enolase. This protein was detected at every stage of the parasite life cycle, in cytosol (the liquid found inside cells) and associated with nucleus, food vacuole (storage membrane bound organelle), cytoskeleton and plasma membrane. Diverse localization of enolase suggests that apart from catalysing the conversion of 2-phosphoglyceric acid into phosphoenolpyruvate in glycolysis (its typical role in cytosol), it may also be involved in other functions, namely, in red blood cell invasion, food vacuole formation and/or development and transcription. These new data seems to clarify the role of this enzyme in the parasite life.

The work of Chugh et al. [55], helped to determine the reasons why two monoclonal antibodies (AC-43 and AC-

29) significantly inhibited *Plasmodium vivax* development inside the mosquito *Anopheles culicifacies* midgut (a portion of insect's digestive system). The gold labeling showed that these two monoclonal antibodies bind to glycoproteins present in the gut epithelium (intestine wall), a known zone for its ookinetes receptors (receptors of the fertilized zygote of the parasite in the mosquito's body), which assures the crossing of the epithelial barrier and provide components for further development into oocysts (the encysted or encapsulated ookinetes in the mosquito's inside). These data point to these glycoproteins as potential candidates for a vector-directed transmission-blocking vaccine, stopping the parasite cycle in the ookinetes–oocysts transition step.

Recently, Lopes da Silva et al. [56] used bovine serum albumin (BSA) linked to 10 nm AuNP in order to follow their position in the parasite–host cell system, in the liver stage, a crucial phase of the *Plasmodium* development. The authors showed that when BSA–Au are loaded into the host cell endocytic pathway, the AuNP are later found within the parasite cytoplasm, showing the transport of materials from the host endocytic pathway towards the parasite interior, revealing a possible form of the parasite to obtain nutrients.

Detection methods using nanogold

Stevens et al. [57] described a different approach using AuNP. The Immunoglobulin M (IgM) antibody bounded to the assay membrane acts as the capture molecule. The gold–antibody conjugate acts as the label, generating a visible increase in optical density proportional to the concentration of analyte present, that is, the malarial antigen *P. falciparum* histidine-rich protein II (PfHRP2). If this antigen (which acts as a bridge between the membrane–IgM and Au–IgG systems) is not present, Au–IgG will disappear after washing and no changes in optical density will be observed. A positive test is schematized in Fig. 6.

Amperometric immunosensors can also be used for monitoring this disease. Sharma et al. [58] presented recently a sensor based on AuNP/alumina sol–gel modified SPE for antibodies to PfHRP2. The AuNP electrode (AuNP/Al₂O₃ sol–gel/SPE) had much larger amperometric current (390 nA) than the bare SPE (120 nA) and Al₂O₃ sol–

gel/SPE (154 nA). AuNPs/Al₂O₃ sol–gel/SPE was three times more sensitive compared to unmodified/bare SPE for the same concentration of analyte. Recently, Fu et al. [59] developed a device consisting on a two-dimensional paper network signal-amplified immunoassay for malaria protein PfHRP2 detection. The paper card contains reagents stored in dry form, including an antibody conjugated to a gold particle label, and the user only needs to add water and the sample. Factors like the signal/noise ratio and test sensitivity are always a concern with AuNP based (and other) tests. Therefore, improvements in this area are also important, besides the test principle itself, as demonstrated for the PfHRP2 malaria biomarker [60].

Potipitak et al. [61] created a device specifically for *P. falciparum* that can be adapted for other parasites. A biotinylated probe was linked to the gold electrode of quartz crystal microbalance (QCM) surface based on the specific interaction between avidin protein and biotin (vitamin B7). Prior to this immobilization, the QCM surface was pre-treated (e.g., with MPA) to add the ester group on the surface, in order to bind with the amine group of avidin. This technique was based on DNA piezoelectric biosensor using the Au electrode of QCM. The deposited mass, due to DNA hybridization on the QCM surface, resulted in a shift of the quartz resonance frequency. Moreover, the new sensor is cost-effective since both sides of the quartz Au surface can be used separately, reducing the sensor price to 50 %.

Very recently, Guirgis et al. [62] described a homogeneous assay based on the fluorescence quenching of cyanine 3B (Cy3B)-labeled recombinant *P. falciparum* heat shock protein 70 (PfHsp70) upon binding to AuNP functionalized with an anti-Hsp70 monoclonal antibody. Upon competition with the free antigen, the Cy3B-labeled recombinant PfHsp70 is released to solution resulting in an increase of fluorescence intensity. Concerning test kits involving colloidal gold, Piper et al. [63] recently showed that a panel of monoclonal antibodies against *Plasmodium* lactate dehydrogenase can be used in various combinations to uniquely identify all species of malaria parasites that infect humans. Their results should help the development of new test with greater specificity, sensitivity and ability to differentiate among malaria parasite species, adding to some solutions already in the market [64].

Nanogold chemotherapy

Concerning antimalarial gold compounds, and in context of the already mentioned series of seven papers related to metal-based chemotherapy against tropical diseases [33–39], Navarro et al. described the reaction of AuPPh₃Cl with chloroquine (CQ) and KPF₆ leading to the new

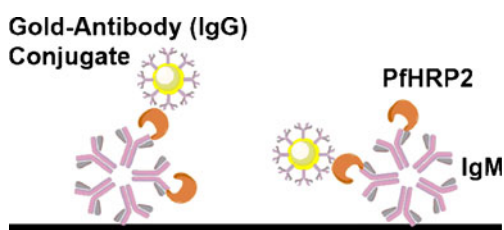


Fig. 6 PfHRP2 positive assay (adapted from [57])

complex $[\text{Au}(\text{PPh}_3)(\text{CQ})]\text{PF}_6$. This compound was found to be considerably more active than CQ diphosphate and other previously reported metal–CQ complexes (Ru or Rh [34]) against two chloroquine-resistant strains of *P. falciparum* in vitro [35]. Navarro et al. also prepared a series of new Au(I) and Au(III) complexes containing CQ in combination with other ligands, which display activity against CQ-sensitive but also against CQ-resistant strains of *P. falciparum* [39]. The highest activity for this series was obtained for $[\text{Au}(\text{CQ})(\text{PEt}_3)]\text{PF}_6$. More recently, Navarro et al. [65] studied the mechanism of antimalarial action of $[\text{Au}(\text{CQ})(\text{PPh}_3)]\text{PF}_6$. This compound seems to avoid heme aggregation (which allows accumulation of toxic levels of compound, resulting in parasite death). The high activities observed against parasites resistant to chloroquine are probably due to the structural modification of CQ introduced by the presence of the gold-triphenylphosphine fragment. The scheme of these complexes can be found in Fig. 7.

Blackie et al. [66] described the synthesis of $[\text{Au}(\mathbf{R})(\text{PPh}_3)]\text{NO}_3$ and $[\text{Au}(\text{C}_6\text{F}_5)(\mathbf{R})]$ complexes, where \mathbf{R} denotes a CQ group or a ferrocenyl-4-amino-7-chloroquinoline, and their use as anti-malarial agents. Rh complexes were also tested. The CQ complexes showed better efficacy against CQ-resistant strains of the *P. falciparum* when comparing to free CQ. For the CQ-resistant strain, there is a considerable drop in efficacy. Concerning to the compounds with the ferrocenyl-4-amino-7-chloroquinolines, those containing ferroquine were considered the most efficient, but all showed improved efficacy with respect to CQ in both sensitive and resistant strains.

Fricker et al. [67] used a set of six Au(III) complexes, showing that all of them were able to inhibit cathepsin B, with a half maximal inhibitory concentration (IC_{50}) values in the range of 0.2–1.4 μM . Cysteine proteases cathepsin B play multiple roles in the parasite life cycle like nutrition, host invasion, protein processing, and evasion of the host immune response, so their inhibition can be extremely important.

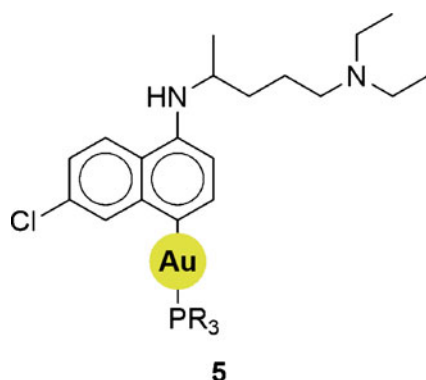


Fig. 7 $[\text{Au}(\text{chloroquine})(\text{PR}_3)]\text{PF}_6$ complexes ($\text{R} = \text{Me}, \text{Et}$ or Ph) prepared by Navarro et al. (adapted from [39, 65])

Also well-known gold-based drugs like Auranofin (the antiarthritic medicine that also demonstrated potential antileishmanial activity, as referred above), showed recently very pronounced antiplasmodial effects in vitro [68]. Auranofin proved to be a potent inhibitor of mammalian thioredoxin reductases causing severe intracellular oxidative stress. Given the high sensitivity of *P. falciparum* to oxidative stress, the authors thought that Auranofin might act as an effective antimalarial agent and showed that this compound (and a few other related gold complexes) strongly inhibits *P. falciparum* growth in vitro.

Recently, Bjelosevic et al. [69] prepared a set of gold(I) complexes based on 1,10-bis(diphenylphosphino) metallocene derivatives (Fig. 8) and evaluated their activity against malaria (W2 chloroquine-resistant strain of *P. falciparum*) and other diseases. Although the IC_{50} values (half-maximal inhibitory concentrations, which indicate how much are needed to induce inhibitions of 50 %) are low, they are still much higher than those for chloroquine. The biological activities of ruthenocenyl-based gold compounds are superior to their ferrocenyl analogues and thus provide directions as to which of these ligands can be used for medicinal applications.

Very recently, Hemmert et al. [70] described the use of gold(I) and gold(III) complexes with *N*-heterocyclic carbene (NHC) ligands as antimalarial agents, specifically against the chloroquine-resistant *P. falciparum* strain FcM29-Cameroon. A group of four dinuclear gold(I) complexes plus three dinuclear gold(III) complexes (Fig. 9, top) was studied, followed by another group of three mononuclear gold(I) complexes (Fig. 9, bottom). The gold(I) dinuclear complexes were found to have moderate antimalarial effect (IC_{50} never lower than 9 μM), but less efficient than the silver(I) counterparts. However, all gold complexes were found to not cause hemolysis (red blood cells membrane bursting with subsequent release of hemoglobin), in contrast to some of the silver analogues. The second group of gold complexes (mononuclear gold(I)) revealed an improved

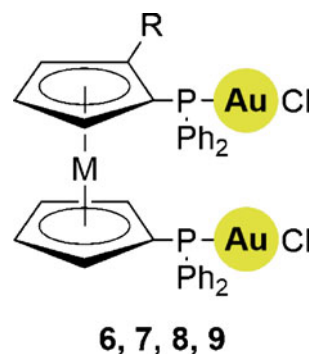


Fig. 8 Au^{I} complexes based on metallocene derivatives prepared by Bjelosevic et al. and tested as antimalarial agents (**6**: $\text{M} = \text{Ru}$, $\text{R} = \text{CHCH}_3\text{N}(\text{CH}_3)_2$; **7**: $\text{M} = \text{Fe}$, $\text{R} = \text{CHCH}_3\text{OCOCH}_3$; **8**: $\text{M} = \text{Ru}$, $\text{R} = \text{CHCH}_3\text{OCOCH}_3$; **9**: $\text{M} = \text{Ru}$, $\text{R} = \text{CHCH}_3\text{NHCO}(\text{CH}_2)_2\text{COOH}$) (adapted from [69])

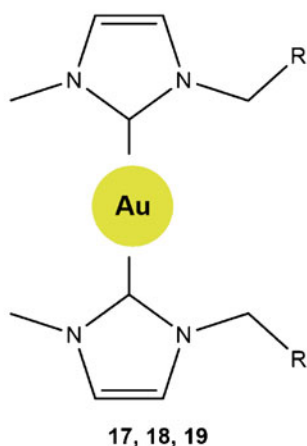
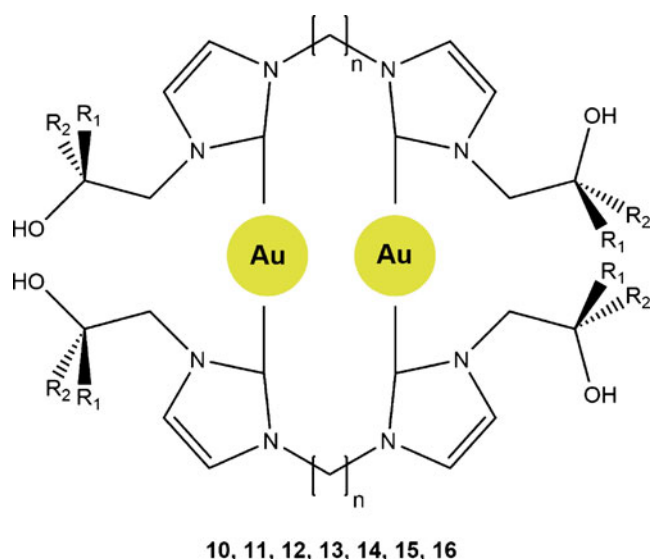


Fig. 9 Dinuclear Au^{I} (**10**: $n=1$, $R_1=R_2=\text{CH}_3$; **11**: $n=2$, $R_1=R_2=\text{CH}_3$; **12**: $n=2$, $R_1=\text{Ph}$, $R_2=\text{H}$; **13**: $n=3$, $R_1=\text{Ph}$, $R_2=\text{H}$), dinuclear Au^{III} (**14**: $n=1$, $R_1=R_2=\text{CH}_3$; **15**: $n=2$, $R_1=\text{Ph}$, $R_2=\text{H}$; **16**: $n=3$, $R_1=\text{Ph}$, $R_2=\text{H}$) and mononuclear Au^{I} (**17**: $R = \text{CONHPh}$; **18**: $R = \text{quinoline}$; **19**: $R = 2,2'$ -bipyridine) complexes (adapted from [70]). Note: for simplicity, the following are omitted in the figure: two PF_6^- counterions for each one of the **10–16** complexes, two Br atoms coordinated to each Au atom for the complexes **14–16** and one Cl^- , PF_6^- or Br^- counterion for complexes **17–19**, respectively

performance compared to the first group, achieving IC_{50} values as low as $0.33 \mu\text{M}$ for one of the compounds.

Soni and Prakash [71] described the larvicidal effect of AuNP (synthesized using biomass derived from the *Chryso sporium tropicum* fungus and HAuCl_4) against *Anopheles stephensi* larvae, a primary mosquito vector of malaria. The exposure to AuNP solutions can lead up to 100 % mortality, depending on the larval instar (developmental stage). Although this study is not a therapy protocol for the disease itself, the larvicidal effect obtained is significant and can lead a better, environmentally safer and greener approach to control this malaria vector in early stages.

Dengue

About the disease

Dengue is a febrile illness that affects infants, young children and adults, in tropical and sub-tropical areas of the world. It is transmitted through the bite of an *Aedes* mosquito, infected with any of the four types of dengue viruses. The symptoms include fever, with severe headache, pain behind the eyes, muscle and joint pain, and rash. They usually appear 3 to 14 days after the insect bite. Severe dengue (plasma leakage, strong hemorrhages, organ failure) is a potentially lethal complication, affecting both children and adults. Naturally, early clinical diagnosis can increase survival of patients [5].

Microscopy assays using nanogold

The reports on the use of microscopy in connection with nanogold, dealing with dengue virus itself, are scarce, probably due to the viral nature of the pathogen, in contrast to the cellular protozoans agents of the other diseases described above. Two works, performed about two decades ago, used immunogold applied to intracellular localization of a dengue antigen:

Thet and Thein [72] showed the potential use of protein-A–Au complex as a marker in the detection of dengue antigen by immunogold labeling for light microscopy. Dengue 4 prototype virus H241, propagated in C6/36 mosquito cell cultures, was used for in vitro experiments. The dengue antigen was detected by using polystyrene beads coated with anti-dengue IgG. The presence of dengue antigen was shown by the formation of pink color (seen with the naked eye) on the beads when incubated with antigen, antibody and protein-A–Au complex. The intensity of the pink color was found to increase with the degree of infection on the infected coverslip cultured cells. The results obtained demonstrated that the use of the protein-A–Au complex had increased sensitivity and was able to detect antigen in the tissue culture at the post infection as early as the first day.

Chen et al. [73] also used immunogold labeling, emphasizing its high sensitivity. A protein-A–Au–Ag staining was used to detect the virus antigens in cultured dengue inoculated C6/36 clone of *Aedes albopictus* cells and human endothelial cells. Data from direct immunofluorescence antibody (DFA) test were compared to immunogold labeling. The study revealed that all DFA-positive specimens were also found positive for immunogold labeling, but not vice versa. This showed that the method using nanogold was more sensitive than DFA.

In a recent study, Vancini et al. [74] analysed the early events in the infection process of dengue and other *Flavivirus*, using electron microscopy and immunogold labeling of viral particles during cell entry. The obtained data supports

the hypothesis of cells infection by a mechanism that involves direct penetration of the host cell plasma membrane.

Detection methods using nanogold

Like in other biosensors described for the previously mentioned diseases, gold can be in the bulk (surface) form, being a support for the sensor assembling active part, as described by Kumbhat et al. [75] for serological diagnosis of dengue virus infection, using surface plasmon resonance (SPR) as analytical technique.

Gold can also be in the form of nanoparticles, as in the following examples: Hsu and co-workers [76] described a sensitive AuNP based inductively coupled plasma mass spectrometry (ICP-MS) amplification and magnetic separation method for the detection of oligonucleotide virus-specific RNA sequences. There is a sandwich-type binding of two designed probe sequences (as illustrated in Fig. 10) that specifically recognize the target regions. They have attached (1) magnetic beads for easy separation and (2) AuNP based beads for ICP-MS amplification detection. Compared with the standard methodology (plaque assay) for the quantification of dengue, the method described allows early detection of the virus in complicated and small-volume samples, with high specificity, good analytical sensitivity, and superior time-effectiveness. The process can be described in five main steps: (1) virus lysing, (2) incubation with magnetic probe and washing, (3) incubation with AuNP probe and washing, (4) releasing and dissolving AuNP for (5) ICP-MS analysis.

Nascimento et al. [77] described the preparation and characterization of a novel gold nanoparticle–polyaniline hybrid composite (AuNP/PANI, schematized in Fig. 11), containing SH terminal groups with the ability of immobilizing dengue serotype-specific primers (ST1, ST2 and ST3). Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were performed. The authors showed that AuNP/PANI-ST(1–3) systems (Fig. 12, left) are capable of detecting dengue serotypes with high specificity and reproducibility at picomolar concentrations. The AuNP/PANI-ST system

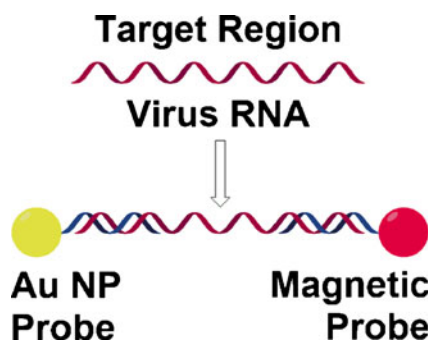


Fig. 10 Sandwich-type binding of two probes to the target region of the RNA virus (adapted from [76])

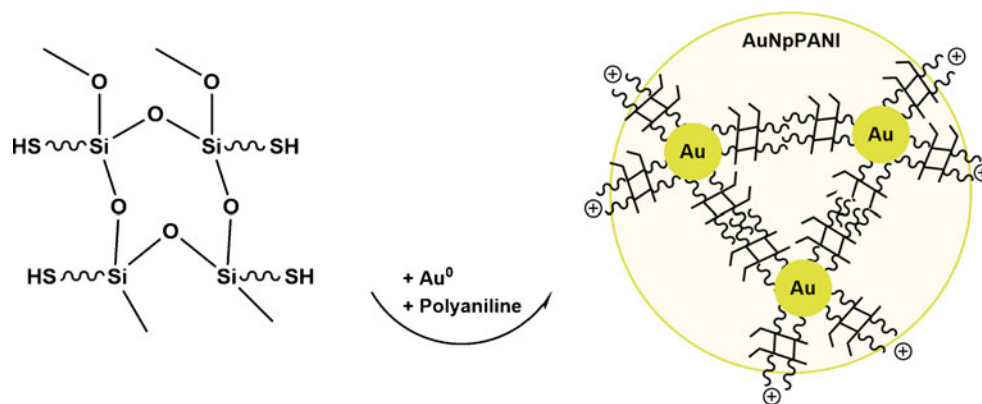
exhibited a highly selectivity response to the complementary target of human patient's dengue genome (Fig. 12, right). According to the authors, this can be a step in development of dengue serotype biosensors that are functional even in the presence of small volumes and low concentrations of the analyte. Even in those conditions, the CV and EIS results showed unequivocal evidence of an existing interaction between dengue serotype-specific primers and their complementary genomic DNA targets.

The dengue IgM capture ELISA is the immunoenzymatic system recommended by the Pan American Health Organization and the World Health Organization for the serological diagnosis of dengue virus infection, due to its high sensitivity, ease of performance, and use of a single acute-phase serum sample [78]. However, tests with the ELISA system are time-consuming and require equipment for washing, incubation, and reading of the results. In 2003, Vazquez et al. [78] described the use of AuBioDOT, a multistep visual diagnostic immunoassay that uses technology based on the IgM capture ELISA principle. This system uses white polyethylene opaque plates as the solid phase, colloidal gold as the marker, and silver ion amplification. It does not require special equipment, it is totally manually operated, and it can be performed in less than 1 h. The application of AuBioDOT for the detection of anti-dengue virus IgM antibodies is recommended as an alternative method for the diagnosis of dengue virus infection, both for clinical diagnosis and for seroepidemiological surveillance. The system is useful under field conditions and in laboratories and requires little equipment. The AuBioDOT IgM capture test for the detection of anti-dengue virus antibodies is a multistep immunoassay that uses manual operation, visual reading, and colloidal gold-labeled conjugated monoclonal antibodies. Colloidal AuNP were used to make a microfluidics-based bioassay that is able to recognize specific DNA sequences via conformational change-induced fluorescence quenching. In this method, a self-assembled monolayer of AuNP was fabricated on the channel wall of a microfluidic chip, and DNA probes were bonded to the monolayer via thiol groups. This test was applied for the detection of the PCR product of dengue virus and results indicate that the assay is specific for the target gene [79].

Oliveira et al. [80, 81] immobilized concanavalin A lectin on gold electrode using AuNP and polyvinyl butyral and put the biosensor in contact to sera from patients infected by dengue. Changes possible to be detected by cyclic voltammetry and electrochemical impedance spectroscopy occurred.

As Foguel et al. [29] suggested for *T. Cruzi* detection, Cavalcanti et al. [82] also used a gold film electrode obtained from a recordable compact disk, but for the detection of non-structural protein 1 (NS1) of the dengue virus. This protein is abundantly present in blood during the acute phase of the dengue infection, in a correlation with viremia

Fig. 11 Schematic representation of AuNpPANI synthesis (adapted from [77])



levels and, consequently, can be used to early diagnostic of the dengue hemorrhagic fever. These disposable gold electrodes with anti-NS1, successfully immobilized onto gold film surface via protein-A, present a high sensitivity (linear response from 1 to 100 ng_{NS1}/ml) with a relatively low detection limit (0.33 ng_{NS1}/ml) and selectivity in a electrochemical label-free detection technique. This same protein (NS1) was also the object of the study of Muller et al. [83], using microprojection arrays, a surface where are distributed micrometric cones or other shapes with sharp points, capable of easy skin penetration (with more than 20,000 projections/cm²). They are usually applied to skin for the purpose of delivering high molecular weight compounds which cannot be delivered efficiently by other means, but used here for directly extracting circulating protein biomarkers from the skin. The use of gold on microprojection arrays deals with their coating in order to allow a better grafting (eventually with help of other small linking molecules) of biomolecules like anti-NS1 antibody for NS1 capture in the subject skin epithelia extracellular fluid. The detection of captured NS1 is made using an anti-NS1 antibody conjugated to HRP, using the principle described in Fig. 2, however with a support–antibody–antigen–antibody HRP assembling instead of the support–antigen–antibody–antibody HRP. This test surpasses others commonly used (like ELISA), which only accept serum/plasma samples, thus requiring significant laboratory-based processing and more invasive procedures for blood collecting.

There are other processes involving nanogold for dengue research and detection, but only slightly different than the several techniques already mentioned: Andrade et al. [84] showed an assembly consisting of a AuNpPANI on a gold

electrode, as already shown in Fig. 12; however, this system recognizes dengue glycoproteins instead of DNA specific regions. Chen et al. [85] used a QCM similar to a malaria test mentioned above, but supporting DNA dengue specific probes and also using a signal amplification technique with AuNP probes as those shown in Fig. 10. Oliveira et al. [86] also used the principles described in the last paragraph, but employed a different lecitin (from *Cratylia mollis* seeds) and Fe₃O₄ nanoparticles.

Unfortunately, until the time this review was written, to the best of our knowledge, there were no reports on the use of gold for dengue therapy, being this an important area of future research.

Conclusions

The results reported above strongly evidence the important role of gold in tropical diseases research. In terms of *imaging*, gold has high electron density which increases electron scatter enabling high contrast images, allowing a new understanding into the molecular immune pathogenesis of these maladies. Immunogold techniques contributed so far to understand the hemoglobin destruction, a critical aspect in the leishmaniasis disease, and also to the localization of an important malaria parasite protein. In terms of dengue, the combination of immunogold labeling and electron microscopy allowed the detection of virus antigens, and to obtain data supporting a mechanism of cells infection by direct penetration of the host cell plasma membrane.

To what concerns the *detection* of tropical diseases, important findings have also been achieved with gold. The research has been growing and several sensors can now assure easier diagnosis, namely in places where the comfort and resources of the laboratory are absent. As described above, gold can be in the bulk (surface) form, being a support for the sensor assembling active part, or in nanoparticles. Au electrodes showed advantages (as they are not influenced by oxygen dissolved in solution) for



Fig. 12 Schematic representation the AuNpPANI on a gold electrode biosensor assembling (adapted from [77])

Chagas disease detection, compared to Pt analogues. For sleeping sickness, one parasite in 180 μL of blood was still detectable in laboratory settings using gold as a colorimetric indicator, which is a remarkable result. Promising test kits using colloidal gold were reported for leishmaniasis and malaria. For the former, a novel cheaper and simplified SELEX procedure using colloidal gold has been reported. Interesting amperometric immunosensors containing gold were used for monitoring malaria. For dengue detection, a fast, fully manual, visual diagnostic immunoassay comprising the IgM capture ELISA principle, with colloidal gold as marker, was reported as an alternative to conventional ELISA systems, which are time-consuming and require a large quantity of equipment. The use of gold on microprojection arrays also surpasses ELISA. Several sensitive AuNP-based tests have also been reported for the efficient early detection of the dengue virus in complicated and small-volume samples.

In terms of disease *therapy*, gold complexes, like the antiarthritic drug Auranofin, showed potential antileishmanial and antimalarial activity. Other gold complexes are promising chemotherapeutic alternatives in the search for the cure of several tropical diseases, namely, $\text{AuCl}_3(\text{CTZ})$ against Chagas disease, $[\text{Au}(\text{dppz})_2]\text{Cl}_3$ for leishmaniasis, $[\text{Au}(\text{PPh}_3)(\text{CQ})]\text{PF}_6$ and other Au(I) and Au(III) complexes containing CQ and NHC ligands for malaria. The latter were found to not cause hemolysis, in contrast to some silver analogues. AuNP also have been reported to have interesting roles in therapy, namely against *Leishmania* promastigotes in culture medium, becoming promising for flexible “band-aids” to be used skin lesions. AuNP also showed an interesting larvicidal effect for a mosquito vector of malaria, which can be an encouraging approach to control the malaria vector in early stages.

In terms of *vaccines*, the role of gold in biolistic immunization using a stable DNA–gold precipitate, not needing a cold chain, is also an attractive method for vaccination against Chagas disease in rural areas. Gold labeling showed that monoclonal antibodies bind to glycoproteins, pointing to these as potential candidates for a vector-directed transmission-blocking malaria vaccine, stopping the parasite cycle in the ookinetes–oocysts transition step.

After all that has been investigated on this topic, the overall conclusion is that the potential of gold for stimulating research in human tropical diseases is considerable. New results will certainly come out soon and lead to even more practical and commercial applications, the full extent of which has still to be envisaged.

Acknowledgments The authors are grateful to the financial support for this work provided by projects PTDC/QUI-QUI/100682/2008 and PESt-C/EQB/LA0020/2011 financed by FEDER through COMPETE,

and by Fundação para a Ciência e a Tecnologia (FCT), and for CIENCIA 2007 program (for SACC).

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