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In vitro biological evaluation of anti-diabetic activity of organic–inorganic hybrid gold nanoparticles

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Kuttalam Sambamoorthy Uma Suganya[1], Kasivelu Govindaraju[1] ✉, Chitoor Veena Vani[1], Mariappan Premanathan[2], Vijaya Kumar Ganesh Kumar[1]

[1]*Centre for Ocean Research, Sathyabama Institute of Science and Technology, Chennai-600 119, India*
[2]*Central Bioscience Research Laboratories (CBRL), Department of Biology, College of Science, Al-Zulfi, Majmaah University, Kingdom of Saudi Arabia*
✉ *E-mail: govindtu@gmail.com*

Abstract: Diabetes mellitus has been considered as a heterogeneous metabolic disorder characterised by complete or relative impairment in the production of insulin by pancreatic β-cells or insulin resistance. In the present study, propanoic acid, an active biocomponent isolated from *Cassia auriculata* is employed for the synthesis of propanoic acid functionalised gold nanoparticles (Pa@AuNPs) and its anti-diabetic activity has been demonstrated in vitro. In vitro cytotoxicity of synthesised Pa@AuNPs was performed in L6 myotubes. The mode of action of Pa@AuNPs exhibiting anti-diabetic potential was validated by glucose uptake assay in the presence of Genistein (insulin receptor tyrosine kinase inhibitor) and Wortmannin (Phosphatidyl inositide kinase inhibitor). Pa@AuNPs exhibited significant glucose uptake in L6 myotubes with maximum uptake at 50 ng/ml. Assays were performed to study the potential of Pa@AuNPs in the inhibition of protein-tyrosine phosphatase 1B, α-glucosidases, and α-amylase activity.

1 Introduction

Type 2 diabetes mellitus (DM) is a non-transmissible chronic disease characterised by continued hyperglycemia and results in inadequate insulin secretion or insulin resistance or a combination of both [1]. Insulin resistance is the result of impaired β-cell functioning, which is usually associated with abnormal insulin secretion. At present, the main treatment for diabetes involves regular uptake of subcutaneous insulin injections and self-monitoring of blood glucose levels which markedly affected the quality of life. Recently, the International Diabetes Federation estimated that diabetic impacts 425 million people in the world [2]. Symptoms of Type 2 DM include lethargy, polyuria, polydipsia and polyphagia among others, type 2 diabetes is associated with some complications such as cardiovascular disease, neuropathy, retinopathy etc. [3]. Despite the availability of diverse drugs for treatment, its efficacy in controlling glucose levels has been limited which requires combinational therapy with different mechanisms of action in order to control glucose levels [4]. Hence, there is a need for alternative therapy that could overcome the limitations prevailing in other treatments.

At present, nanoscience applications have provided a novel platform for target-specific delivery of therapeutic agents. Over the last decade, nanoparticles have gained increasing attention due to their promising applications in drug delivery. Several studies have demonstrated the use of different types of nanoparticles, in particular, gold nanoparticles (AuNPs) for insulin delivery mainly through transmucosal or transdermal routes to improve the glycaemic response to glucose challenge for an extended period of time [5]. Various metal and metal oxide nanoparticles have been used and proven to possess significant anti-diabetic activity in comparison with commercial drugs [6–11]. Functionalisation of AuNPs was found to be more active to interact with their specific targets and enhance their mode of action. AuNPs synthesised using gymnemic acid, a secondary metabolite of *Gymnema sylvestre* have demonstrated enhanced glucose utilisation/uptake efficiency of 3T3-L1 (preadipocytes) adipocytes through the suggested insulin-dependent/-independent pathway [12]. There are studies that demonstrate the prolonged effect of insulin-coated AuNPs on glucose regulation which can potentially be used for personalised diabetic treatment [13]. The potential for clinical implementation of AuNPs has led to substantial research on their in vivo chemical stability, pharmacokinetics, biodistribution, and bio-toxicity. Herein, AuNPs have been chosen as a model system for numerous unique advantages including greater flexibility in terms of particle size, shape, a high degree of surface functionalisation, longer shelf-life period, and biocompatibility [14–16]. Therefore, functionalisation of AuNPs for anti-diabetic studies gained attention. In the present study, propanoic acid functionalised AuNPs were synthesised and their in vitro anti-diabetic efficacy was evaluated.

2 Materials and methods

2.1 Synthesis of AuNPs

Propanoic acid-functionalised AuNPs (Pa@AuNPs) were prepared following our previous protocol published earlier [17]. Briefly, propanoic acid (600 mg) was dissolved in 45 ml of distilled water and mixed with 5 ml of 1 mM aqueous auric chloride ($HAuCl_4 \cdot 3H_2O$) solution. The observed colour change from pale yellowish brown to ruby red indicates the formation of Pa@AuNPs and stored for further studies.

2.2 Cell culture and development of insulin resistant model using L6 myotubes

L6 cells were procured from the National Centre for Cell Science, Pune, India. The cell culture was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) and supplemented with an antibiotic and antimycotic solution (Himedia) in a CO_2 environment [18]. For the insulin resistant state, the growth medium of L6 cells was replaced by maintenance medium (DMEM with 2% FBS) for 4–5 days post confluence. Observing the differentiation by multi-nucleation of cells, differentiated cells were treated with high-glucose medium (25 mM/l glucose) for 24 h. After incubation, the cells acquire an insulin resistant state, which is used for in vitro experimentation [19].

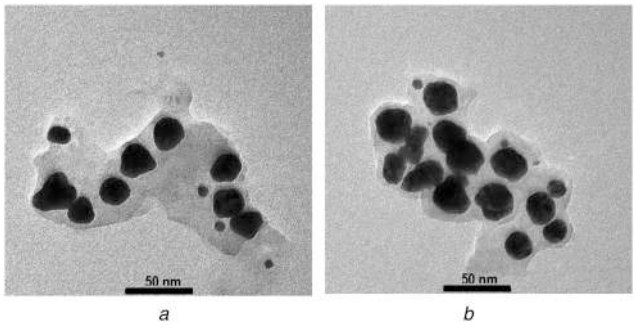

Fig. 1 *TEm image of Pa@AuNPs*
(a) Pa@AuNps aggregates are not formed, ***(b)*** Monodispersity of the Pa@AuNps wih uniform size

2.3 Glucose uptake measurements

Insulin resistant L6 myoblasts cells were cultured in 24-well plates and the cells were treated with various concentrations of Pa@AuNPs for 24 h and glucose uptake was corrected for non-specific uptake in the presence of 10 µM cytochalasin B. Rosiglitazone was used as positive control. The assay was carried out in triplicate and the results were expressed as glucose uptake (%). For inhibitor studies, L6 cells were treated with Genistein (50 µM) and Wortmannin (100 nm) [20, 21], 30 min prior to incubation with Pa@AuNPs followed by glucose uptake assay.

2.4 In vitro protein-tyrosine phosphatase (PTP1B) inhibition assay

In vitro PTP1B inhibitory activity was carried out by a calorimetric method using the PTP1B assay kit (Calibiochem, Merck Cat. No. 539736) as per manufacturer's protocol.

2.5 α-amylase and α-glucosidase inhibition assay

For α-amylase inhibition assay, different concentrations of Pa@AuNPs were incubated with α-amylase (50 mg ml^{-1}) for 30 min at 37°C. Starch solution (1%) was used as a substrate, samples without α-amylase were taken as control and test readings were subtracted from the absorbance of the control. Reducing sugar was estimated by interpolating the obtained absorbance value of 3,5-dinitrosalicylic acid for different concentrations of Pa@AuNPs at 540 nm [22]. The percentage of enzyme inhibition was calculated using the following formula:

$$\%\text{Enzyme inhibition} = \text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}\text{Abs}_{\text{sample}} \times 100\,.$$

For α-glucosidase inhibition assay, 100 ml of α-glucosidase (0.1 U/ml) was added to 200 ml of different concentrations of Pa@AuNPs and incubated for 1 h at 37°C. Enzyme action was initiated by the addition of 10 mM p-nitrophenyl-a-D-glucopyranoside (p-NPGP) in phosphate buffer (100 mM) of pH 6.8 and the reaction was stopped after 10 min, incubated at 37°C by adding 2 ml of Na_2CO_3 (0.1 M). Determination of α-glucosidase activity was carried out by estimation of absorbance by p-nitrophenol released from p-NPGP at 420 nm [23]. % Enzyme inhibition = $\text{Abs}_{\text{sample}}$–$\text{Abs}_{\text{control}}$ /$\text{Abs}_{\text{sample}}$ × 100.

2.6 Toxicity studies

In vitro cytotoxicity assay was carried using (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) assay [24]. L6 myoblasts cells (1×10^5 cells/well) seeded in a 96-well microtitre plate with a growth medium and cultured for 4 days. The medium was replaced and incubated with different concentrations of Pa@AuNPs for 24 h. The treated cells were washed with phosphate-buffered saline and then incubated with 10 µl of MTT for 4 h. Formazan crystals were solubilised in 100 µl of dimethylsulphoxide and measured at 570 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek, Winooski, VT, USA).

2.7 Statistical analysis

All the experiments were conducted in triplicate. Results were presented as mean with standard errors. Assays involving treatment with a single drug were analysed by one-way analysis of variance (ANOVA) with Turkeys multiple comparison tests. Assays with more than one drug were analysed using two-way ANOVA with Bonferroni post-test. Differences between groups were regarded as significant at a probability error (P).

3 Results and discussion

3.1 Transmission electron microscopy (TEM) image of Pa@AuNPs

From TEM images, it could be observed that the nanoparticles formed were monodispersed, without any agglomeration with an average size of ∼35 nm (Figs. 1*a* and *b*).

3.2 Glucose uptake assay and protein-tyrosine phosphatase B inhibitory activity

Pa@AuNPs were subjected to a glucose uptake assay, a dose-dependent increase in glucose uptake activity was observed (Fig. 2*a*). 50 ng/ml of Pa@AuNPs was observed to be the optimal dose showing maximum activity. Glucose transport behaviour of Pa@AuNPs in the presence of the insulin receptor tyrosine kinase (IRTK) inhibitor (Genistein, 50 µM) and Phosphatidyl inositide kinase (P13K) inhibitor (Wortmannin, 100 nM) at the concentration indicated was subjected to glucose uptake assay (Figs. 2*b* and *c*). Inhibitors suppressed the insulin-mediated glucose transport but did not control the glucose uptake activity of Pa@AuNPs. Insulin-mediated glucose uptake was negatively regulated by protein tyrosine phosphates. As shown in Figs. 3*a* and *b*, Pa@AuNPs exhibited dose- and time-dependent PTP1B inhibition with IC_{50} of 1.25 µg/ml and maximum inhibition was obtained at 60 min and positive control RK-682 (IC_{50} 5 µM). Both under normal and diabetic conditions, the protein tyrosine kinases play an important role in numerous cellular signalling pathways [25]. It activates the tyrosine kinase by insulin leading to the phosphorylation of its receptors thus inducing a functional change of P13K signalling molecule [26]. Wortmannin inhibits insulin-stimulated P13K and Glucose transporter 4 translocation activity [27], and Genistein reported as THE IRTK inhibitor controls the insulin-stimulated glucose uptake activity [28] was used in the present study. Neither P13K nor IRTK pathways were involved in the mode of action of Pa@AuNPs. Based on our results, it is suggested that the action of Pa@AuNPs is independent. PTP1B is a negative regulator of insulin-mediated glucose uptake and is localised on the cytoplasmic surface of the endoplasmic reticulum in adipose, muscular, and hepatic tissues [29].

Inhibition of PTP1B would lead to reduced levels of plasma glucose and enhance the insulin action. It is ubiquitous in the insulin-targeted tissues and possess the reported role in insulin resistance development [30], inhibiting PTP1B and in the treatment of type 2 diabetes [31]. α-Glucosidases and α-amylase are a complex group of enzymes that hydrolyse chemical bonds of the disaccharides such as sucrose and maltose and generate monosaccharides glucose and fructose. These enzymes are responsible for the transport of carbohydrates in the bloodstream [32].

3.3 α-Glucosidases and α-amylase inhibitory activity

Inhibition of these enzymes (α-glucosidases and α-amylase) is a therapeutic target for the treatment of type 2 DM. Since such inhibition delays/decrease the absorption of carbohydrates lowering the hyperglycemic levels [33], it was aimed to analyse the potential of Pa@AuNPs in inhibiting these enzymes. From the study conducted, it could be observed that Pa@AuNPs inhibited the activity of α-glucosidases and α-amylase in a dose-dependent manner with IC_{50} of 2.35 and 1.76 µg/ml, respectively (Fig. 4).

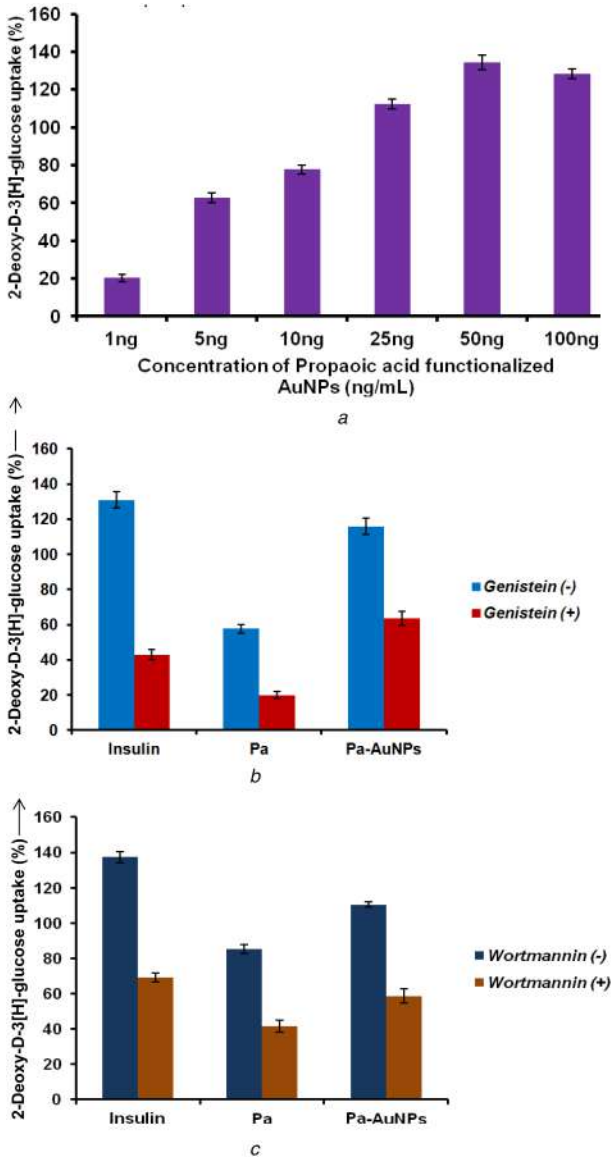

Fig. 2 *Glucose uptake activity assay. Dose response glucose uptake activity of Pa@AuNPs*

(a) Glucose uptake behaviour of Pa@AuNPs in the presence and absence of IRTK inhibitor Genistein, ***(b)*** P13K inhibitor Wortmannin, ***(c)*** Values are expressed as mean ± S.E ($p < 0.05$)

3.4 Cytotoxicity of Pa@AuNPs in L6 myotubes

The cytotoxicity of Pa@AuNPs was examined in L6 cells by MTT assay. From the study, it showed that the CC_{50} of Pa@AuNPs was 225 µg/ml (Fig. 5). AuNPs, due to their unique physical and chemical properties, ease synthesis and surface modifications/ functionalisation have been extensively used in biomedical applications [34]. In the present study, Pa@AuNPs exhibited a potent anti-diabetic activity which is evident from the in vitro assays conducted. The findings demonstrated that Pa@AuNPs inhibited α-glucosidases, α-amylase and PTP1B activities with IC_{50} values of 2.35, 1.76, and 1.25 µg/ml, respectively, i.e. much lower than the cytotoxicity of L6 cells with therapeutic index (i.e. the ratio between CC_{50} and IC_{50}) of 95 which may be of clinical interest.

4 Conclusions

Pa@AuNPs were synthesised and their anti-diabetic activities have been demonstrated in vitro. It was observed that the synthesised AuNPs enhanced the glucose uptake efficiency of L6 myotubes. Glucose transport behaviour of Pa@AuNPs in the presence of IRTK (Genistein) and P13K (Wortmannin) inhibitors suppressed

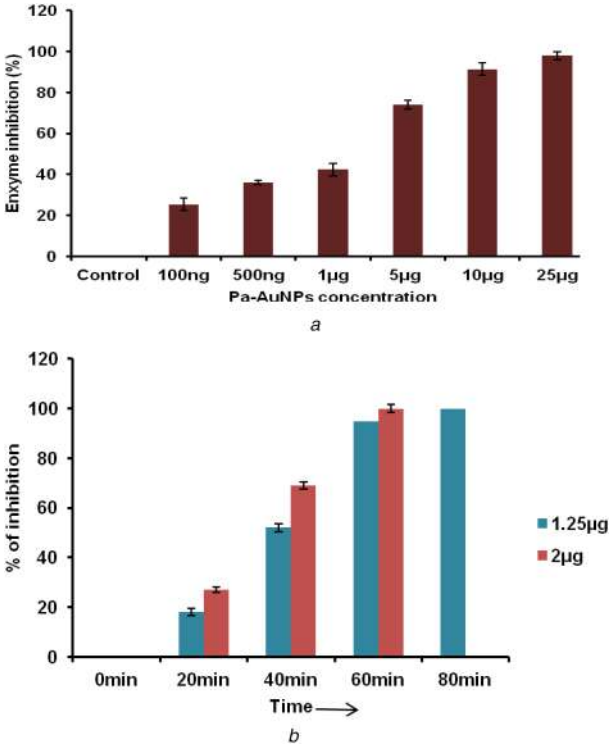

Fig. 3 *PTP1B activity assay. Dose response inhibition of the PTP1B enzyme*

(a) Time response of inhibition of PTP1B, ***(b)*** Values are expressed as mean ± S.E ($p < 0.05$)

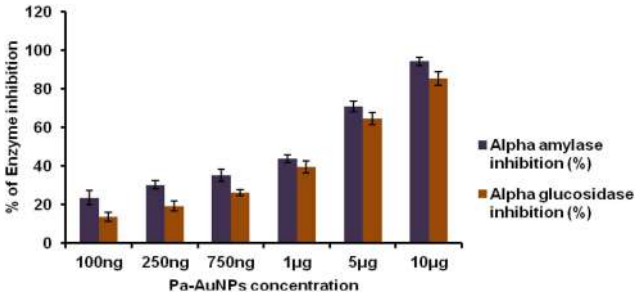

Fig. 4 *Effect of Pa@AuNPs against α-glucosidases and α-amylase. Values are expressed as mean ± S.E ($p < 0.05$)*

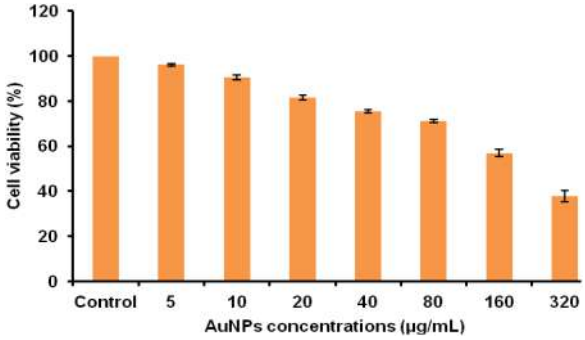

Fig. 5 *Cytotoxicity of Pa@AuNPs in L6 myotubes cells. Values are expressed as mean ± S.E ($p < 0.05$)*

insulin-mediated glucose transport but the inhibitors did not control the glucose uptake activity of Pa@AuNPs. Pa@AuNPs enhanced the glucose uptake efficiency of L6 myotubes cells. Glucose transport behaviour of Pa@AuNPs in the presence of IRTK (Genistein) and P13K (Wortmannin) inhibitors was suppressed in the insulin-mediated glucose transport but the inhibitors did not control the glucose uptake activity of Pa@AuNPs. Also, PTP1B, α-glucosidases and α-amylase activities have been inhibited by Pa@AuNPs, which as the therapeutic targets for the treatment of

type 2 diabetes. Cytotoxicity of Pa@AuNPs towards L6 cells showed CC_{50} of 225 µg/ml. Based on the results obtained it is emphasised that the cytotoxicity and anti-diabetic activity of Pa@AuNPS are more effective.

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