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Multi-spectroscopic investigation on the contradictory relevance of metal nanoparticles in pharmacological milieu of protein glycation and cholinergic inhibition

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Abstract

While unique physico-chemical behaviour of engineered nanoparticles have brought about a large-scale revolution in many fields like medicine, industry, agriculture and technology, it may result in certain toxicity due to unsolicited interactions with diverse biological sections and cellular processes. In this contribution, we report on the inhibitory effect of colloidal gold nanoparticles (AuNPs) towards the formation of Advanced Glycated End (AGE) products, which proves advantageous in the treatment of diabetes mellitus (DM). On the other hand, AuNPs boost the activity of acetylcholinesterase (AChE), the key enzyme which forms the basis of the cholinergic hypothesis for Alzheimer's disease (AD). Since higher activity of AChE increases the severity of AD, this activity-enhancing effect can be considered as nanotoxicity in this context. These seemingly contradictory facets of AuNP interaction with the biological entities associated with two deadly modern-day diseases like DM and AD, are explored in detail.

Key words: Metal nanoparticle; AGE product fluorescence; cholinergic hypothesis; diabetes mellitus; Alzheimer's disease

1. Introduction

The biomolecules responsible for smooth functioning of countless metabolic functions in the body are of nanoscale dimensions. Therefore nanoparticles (NPs), which have gained wide recognition in the past decade for their various applications, make for an interesting system to study nano-bio interactions (Wang *et al.,* 2019; Darr *et al.,* 2017) . The midas touch of nanotechnology has left very few aspects of human life unaffected, and many reports show that NPs exert considerable influence on bodily functions of absorption, distribution, metabolism and excretion (ADME) of life-saving drugs (Yang *et al.,* 2010). They also affect the drug carrying affinity of serum proteins, as well the activity of other biomolecules (Abbasi *et al.,* 2012).

NPs have a plethora of potential innovative applications in many avenues of biomedicine, catalysis, fuel cell, magnetic data storage, agriculture, solar-cell etc (Han *et*

al., 2019). Extensive research has been carried out on a variety of NPs like gold, silver, iron oxide, silica, and carbon-based materials, for diagnostic and therapeutic purposes. NPs loaded with drugs show drug release at precise rates and doses at target sites in the body for specific durations, resulting in accurate transport. This enhances the therapeutic effect of the drug and reduces the toxicity as well as the side effects (Paul and Sharma, 2020). Due to their suitable dimensionality, NPs also possess the ability to cross the Blood Brain Barrier (BBB) (Posadas *et al.,* 2016) further enhancing their usefulness in drug discovery and drug targeting.

Gold nanoparticles (AuNPs) have wide application as biomarkers due to their tunable optical property and are widely used for photo-thermal therapy of cancer cells (Jain *et al.*, 2012). Magnetic NPs, commonly iron oxide nanoparticles, have also been recently used as an alternative for cancer therapy (Saeed *et al.*, 2018). In addition to these, the activity of AuNPs along with their biocompatibility has made them preferable for ophthalmological implications. On a different note, it is well known that oxidative stress plays a significant role in the etiology of several diabetic complications (Giacco and Brownlee, 2010; Samadi *et al.,* 2019). The ability of AuNPs to inhibit the lipid from peroxidation, and thereby preventing the generation of reactive oxygen species (ROS), has restored the imbalances in the antioxidants and liver enzymes which are responsible for the cell dysfunction and destruction in the diabetic control group at hyperglycemic conditions. Thus, AuNPs are also regarded as potent antioxidants (Barathmanikanth *et al.,* 2010).

Typically, smaller NPs favour native-like protein structure, resulting in higher intrinsic enzyme activity of the protein-NP bioconjugate (Kozlowski *et al.,* 2018). The mechanism behind the impact on protein structure is generally explained by a simple model involving the available surface area for varied sizes of NPs. The large surface area of contact for the adsorbed proteins, provided by the relatively bigger sized NPs, results in stronger interactions between proteins and nanoparticles (Saptarshi *et al.,* 2013). This larger degree of interaction leads to greater perturbation in the protein structure.

Serum albumins are proteins which constitute nearly 60% of the blood plasma and play a primary role in the transport of enzymes, minerals as well as drugs in the body. Thus, these carrier proteins provide the most relevant bio-mimicking prototype for analysing the mechanism of drug interactions (Wong and Ho, 2018). It would therefore be interesting to determine the augmented drug carrying capacity of serum proteins quantitatively in presence of colloidal metal NPs. However, incorrect usage of NPs targeting the efficient drug delivery in the body leads to toxic effects. Therefore, an accurate and judicious standardization is required to achieve a nanomaterial showing different properties like enhanced diagnosis, as well as monitoring and treatment of human diseases. It is pertinent to mention here that the use of NPs not only depends on its size and shape, but on a combination of different cellular uptake mechanisms such as varying level of the target receptor, membrane fluidity, and cell cycle, to name a few (Stark, 2011).

Fluorescence-based studies on drug interaction with NP-protein bioconjugate have attracted massive attention in recent years. In these studies, fluorescence quenching is commonly employed to understand the thermodynamic properties of drug binding, number of binding sites etc. Colloidal metal NPs of gold and silver (AuNP and AgNP, respectively) have been shown to increase drug transport capacity and improve drug-protein binding in specific cases, a few reports of which have recently been published by our laboratory (Sonu *et al*., 2019; Sonu and Mitra, 2019). Furthermore, previous experiments on FDA-approved anti-Alzheimer drugs working as AChE inhibitors have revealed that the inhibition potency of the drugs differ considerably in the presence of human serum albumin (HSA), compared to aqueous buffer. HSA significantly reduces the inhibition potency of a series of well-known drugs. This modulatory behaviour of the serum albumin has established it as an important pharmacological medium for studying AChE inhibition efficacies (Islam *et al*., 2016). On the other hand, kinetic enzyme assays are widely employed to mimic relevant biological media to study the effects of NPs on the activity of various enzymes of interest.

In this contribution, we present some of our recent findings on the inhibitory effect of metal NPs on the formation of Advanced Glycated End (AGE) products and their boosting effect on acetylcholinesterase (AChE) enzyme activity. Detailed investigation reveals that the effect of AuNP on glycation is somewhat different from that of an anti-diabetic drug, chlorpropamide (CPM). The effect of AuNP and HSA on the inhibition effect of the FDA-approved AD drug Donepezil (DON) was observed to be analogous, albeit with different mechanisms and degrees of interaction. Before elaborating on the details of the experimental results, we give a brief introduction of the AGE product formation and AChE enzyme activity with reference to two deadly and commonly incurred ailments in modern day human health, namely diabetes mellitus (DM) and Alzheimer's disease (AD). This is followed by a succinct description of the experimental protocols. Finally, we analyse the modulating effect of NPs on these two bio-chemical pathways from a therapeutic point of view.

2. Diabetes Mellitus and formation of Advanced Glycated End (AGE) Product

Hyperglycemia, which refers to a spike in the blood glucose level, is the foremost symptom expressed in diabetic patients (Giacco and Brownlee, 2010). In prolonged hyperglycemia, the aldehyde functional group of the reducing sugar molecules binds to the amino group of the exposed lysine and arginine residues of the protein, leading to the formation of a Schiff base, and thereafter a rearrangement product known as Amadori product (Baynes *et al.,* 1989). Following several steps, combinedly known as Maillard reaction, the formation of advanced glycation end (AGE) products occurs. This entire process is termed as *glycation*. Accumulation of AGE products for an extended time causes many diabetic complications, cataract formation as well as neurodegenerative disorders like Alzheimer's and Parkinson's disease etc (Li *et al.,* 2012; Vicente Miranda *et al.,* 2016).

It is difficult to alter the level of glycated albumins by haemoglobin metabolism. Therefore, the glycated albumins can be used as a short-term measure to gauge glycemic control in diabetic patients, which facilitates its usage as a medical marker for diabetes (Roohk and Zaidi, 2008). Glycation brings about modifications in the structure of serum albumins which in turn alters the enzymatic activity, binding affinity, transportation and distribution of drugs in the body (Anguizola *et al.,* 2013). Many drugs, like metformin and aminoguanidine etc., have been synthesized with promising inhibitory effects towards the glycation of serum albumin (Abbas *et al.*, 2016; Arasteh *et al.*, 2014). However, certain side effects have posed hurdles to their unhindered advancement as a viable therapeutic avenue.

In this regard, the compatibility of NPs with biological systems, as discussed earlier, minimizes the problems related to finding inhibitors of glycation. This is because they can easily pass through the cell membranes and possess a desirably high surface to volume ratio due to which cells, proteins, and biological membranes can be adsorbed easily on the NP surface. Recently ZnO, gold (Au) and selenium (Se) nanoparticles have shown the capacity to inhibit glycation of albumins by D-ribose and glucose (Seneviratne *et al.,* 2012; Liu *et al.,* 2014). In this report, the efficacy of AuNPs (which possess low toxicity and easy bioavailability) in inhibiting the glycation of bovine serum albumin (BSA) induced by L-arabinose, has been quantified and compared with anti-diabetic drug chlorpropamide (CPM) as a reference model system.

3. Cholinergic hypothesis on Alzheimer's disease

Acetylcholinesterase (AChE) is the enzyme involved in terminating impulse transmission through rapid hydrolysis of the neurotransmitter acetylcholine (ACh). Decline in ACh levels has been established as a key element in the pathogenesis of Alzheimer's Disease (AD) (Terry and Buccafusco, 2003). Most of the commercially available drugs currently employed in the treatment of AD are based on this theory, typically known as the *cholinergic hypothesis* (Nordberg, 1992). The modus operandi of the drugs is to increase the concentration of ACh in the synaptic cleft by inhibiting the AChE activity. This subsequently boosts cholinergic neurotransmission in the brain, and thus, AChE inhibition has been established as the most feasible route in the treatment of AD. A direct corollary of this hypothesis is that any substance which reduces the activity of AChE would be considered advantageous in the purview of AD. On the other hand, any entity causing an increase in the activity would be considered toxic in the same regard.

The primary direction in designing and development of AD drugs lies in the search for potent inhibitors of AChE activity, as this leads to a spike in the production of acetylcholine and, consequently, improves neurotransmission. With improved, environmentally benign, and efficient synthetic methodologies in combination with in-silico molecular modeling protocols, the search for novel AChE inhibitors has gained momentum lately. A considerable amount of work in this regard has also been reported by our laboratory (Baruah *et al*., 2019a, 2019b; Rohman *et al*., 2019). In this contribution,

the modulatory AChE inhibition activity of DON in buffer medium and physiologically relevant HSA matrix has been explored in detail. Further, the effect of AuNP on the enzymatic activity of AChE is also reported. The pharmacological consequence, as well as the mode of action in both the cases are discussed in an exhaustive manner.

4. Materials & Methods

All reagents/chemicals used in this study were of highest quality available and purchased from reputed vendors like Sigma-Aldrich, Qualigens, Merck etc. AuNPs (of size 14±2 nm) were synthesized through standard reduction procedure described elsewhere. (Sonu & Mitra, 2019). BSA was glycated by reacting with 0.4 M of L-arabinose wherein the concentration of the working protein solution was kept fixed at 5 μM. A fixed amount of 0.13 nM and 0.68 nM solution of AuNPs was added during the process of glycation to check if AuNPs exerted any influence in the glycation process. The glycated BSA (gBSAara) was incubated for 90 hrs at 37 °C. Excitation at 335 nm is used to monitor protein modifications induced by the monosaccharide's pentosidine type compound; whereas, excitation at 370 nm is used to investigate the alteration in protein due to the formation of different AGE products (Cervantes-Laurean *et al*., 2006). The inhibition of the glycation by AuNPs was examined by observing the AGE's intensity after every 10 hrs.

Absorption measurements were recorded on a double beam, ultraviolet-visible (UV-Vis) spectrophotometer (PerkinElmer Lambda25). Steady state fluorescence spectra and quantum yield measurements were carried out in Quanta Master (QM-40) steady state apparatus obtained from Photon Technology International (PTI). All the fluorescence spectra were corrected for the spectral sensitivity of the photomultiplier tube (PMT).

The acetylcholinesterase enzymatic activity in absence and presence of various inhibitor drugs was measured spectrophometrically in a high-throughput technique following the method originally developed by Ellman and co-workers in 1961 (ELLMAN *et al*., 1961), the details of which have been described elsewhere (Baruah, Basumatary, *et al*., 2019; Baruah, Rohman, *et al*., 2019).

Analysis of enzymatic hydrolysis data using Michalis – Menten (MM) model both in presence and absence of the inhibitor results in the estimation of initial rate (V_0), MM constant (K_m) and maximum hydrolysis rate (V_{max}). The substrate concentration was varied from 50 μM to 2.0 mM. To study the inhibition potency of various inhibitors in all the studied media the substrate concentration was kept fixed at the saturated reaction condition of AChE catalysis reaction (~1.5 mM) and, in some cases, at around the K_m value of the enzyme (0.15 mM) (Khandkar *et al*., 1995); whereas, the inhibitors concentrations were varied from very low till the saturation inhibition condition. In both the non-enzymatic as well as enzymatic reaction conditions, the [DTNB] were kept fixed at ~317 μM; whereas, the enzyme concentrations were ~0.079 units/ml in all the enzymatic reactions on-enzymatic blank was subtracted for each kinetic measurement

and all components were incubated for 5 min on ice cold water bath followed by total run time of 50 min inside the plate reader at the experimental temperature to follow the kinetics.

The well-established Hill protocol (Copeland, 2005) was utilized to study the effectiveness of the various inhibitor drugs towards the AChE enzymatic activity which yielded the values of the decrease in initial velocity in the presence of certain inhibitor concentration (ΔV), the maximum decrease in initial velocity (ΔV_{max}), the concentration of the inhibitor that gives half-maximal initial velocity, $K_{0.5}$ (which is pharmacologically equivalent to IC_{50}), and the Hill coefficient, n_H.

5. Results and Discussion

5.1. Effect of AuNP on the glycation of BSA: Comparing with model anti-diabetic drug

5.1.1. Monitoring AGE product fluorescence. BSA was glycated by incubating the protein with 0.4 M of L-arabinose solution for a period of 48 hours and the AGE'S fluorescence was examined after every 10 hours. It is to be noted here that the concentration of L-arabinose used in this study is substantially higher than that present in the physiological condition (Ning & Segal, 2000) This is to ensure the formation of sufficient and experimentally detectable glycated product in a reasonable time frame. Optimization of the experimental conditions, however, indicates that it is apparently possible to measure the glycation even after 24 hours, which gives adequate signal to noise ratio to quantitatively examine the AGE fluorescence, since the nature of fluorescence spectral profile remains the same even after longer incubation with only an increase in the fluorescence emission intensity.

To comprehend the extent as well as the effect of glycation and formation of AGE products, fluorescence spectrum of the incubated BSA solution was obtained by excitation at 335 nm and 370 nm (Figure 1). Blank data of the buffer and the sugar solution, taken under similar experimental conditions, were subtracted from each spectrum. Further, the solutions of model anti-diabetic drug chlorpropamide (CPM) with varying concentrations (ca. 0.08 and 0.16 mM) were checked to verify if CPM possesses any fluorescence intensity. No significant fluorescence was observed at either of these wavelengths. Excitation of the glycated protein ($gBSA_{ara}$) at 335 nm results in the AGE's fluorescence spectrum at around 415 nm, which is close to the intrinsic emission of the pentosidine molecule. Addition of 0.16 mM concentration of the antidiabetic drug CPM enhances the AGE fluorescence intensity by more than twice in case of $gBSA_{ara}$ (Figure 1a, inset). On the other hand, excitation at 370 nm results in the AGE's fluorescence intensity around 440 nm, which corresponds to the characteristic peak of crossline and vesperlysine. Even with 0.08 mM of CPM added to the glycated protein solutions, an appreciable increase in the AGE fluorescence is observed (Figure 1b, inset).

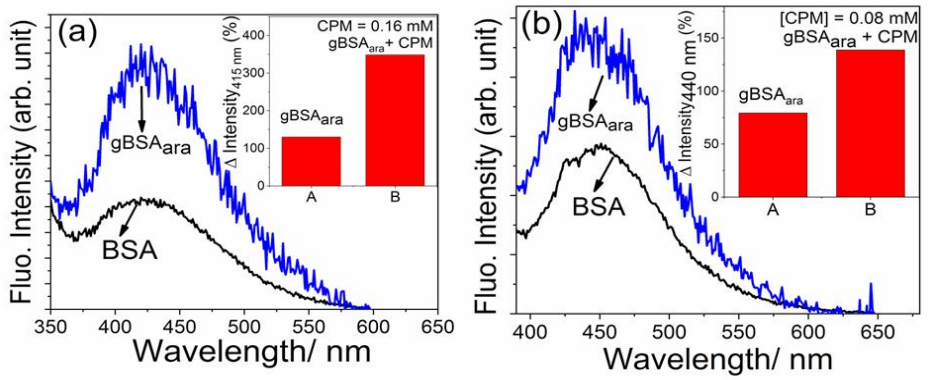

Figure 1: Fluorescence spectra of BSA and L-arabinose induced glycated BSA (gBSAara) excited at 335 nm (a) and 370 nm (b), respectively. Inset shows the bar diagram of AGE's intensity generated (in percentage) relative to that of native BSA when excited at 335 nm and 370 nm respectively.

In presence of colloidal AuNP solution, a sharp diminution is observed in the fluorescence intensity of glycated BSA. The intensity further decreases on increasing the concentrations of AuNP. AGE's fluorescence intensity of gBSAara is reduced by 55% when 0.13 nM of AuNP was added, indicating an inhibition in the development of the AGE's related compounds in presence of AuNP. However, the emission intensity decreases by a significant 80% at [AuNP] = 0.68 nM (Figure 2a). Interestingly, the inhibitory efficacy exerted by AuNPs is observed to decrease over a prolonged time. For example, incubation of 34 hours leads to the decrease in glycated product fluorescence merely by 20% or 40% under the similar concentration of AuNP (Figure 2b). When the excitation wavelength is set at 370 nm, similar effects are also observed as in the case of 335 nm excitation. There is a 49% decrease in the AGE's intensity of gBSAara when incubated with 0.16 nM solution of AuNP, in comparison with 84% decrease at [AuNP] = 0.68 nM. This points towards a concentration and time dependent effect of the AuNPs in the effective inhibition of glycated BSA (Inset, Figure 2a).

These studies show a highly interesting consequence that while CPM increases the extent of protein glycation, AuNPs decrease it significantly. Interestingly, the increase in glycation of serum protein by anti-diabetic drug CPM directly contradicts its therapeutic effectiveness in the purview of diabetes. The clarification perhaps lies in the fact that the modus operandi of CPM is to boost the production of insulin by the beta-pancreas and not to act as an anti-glycating agent (Miller & Moses, 1970). Our studies prove that the presence of an excessive amount of CPM in blood serum either due to protracted usage or drug overdose surges the likelihood of protein glycation and might prove hazardous in the long run.

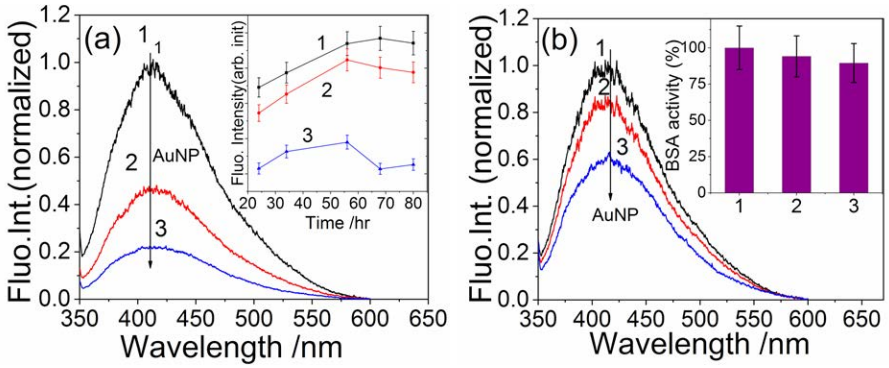

Figure 2. AGE's fluorescence spectra of gBSara (λ_{exc} = 335 nm) after 24 hr (a) and 34 hr (b) incubation with [AuNP] /nM = 0 (1), 0.13 (2) and 0.68 (3). Inset shows the variation in AGE intensity with time in different AuNP concentration (a) and consistent BSA activity at different concentrations of AuNP (b).

Nevertheless, from a pharmacological point of view, AuNP mediated delivery formulation of CPM can be hypothesized to achieve the benefit of both these two pathways. It is expected that this combined formulation would act simultaneously in improving the insulin production through CPM, while inhibiting the AGE product formation through AuNP. However, the possible concerns may be the effect of AuNP on the drug carrying capacity of the serum protein and retention of enzymatic activity of albumin protein – NP bioconjugate.

5.1.2. Interaction of CPM with native protein and protein – AuNP bioconjugate. The binding efficiency of CPM with protein and protein-AuNP conjugate is determined by monitoring the intrinsic tryptophan fluorescence of BSA on varying concentrations of the drug. In both the cases, the fluorescence spectra centred at 342 nm is quenched regularly with an increase in the concentration of the drug without any shift of the emission maxima. Detailed steady state and time-resolved fluorescence analysis along with molecular docking calculation and other complementary spectroscopic investigations confirm that the fluorescence quenching is due to the ground state complex formation between the protein and the drug primarily through an "entropy driven" pathway (Singh & Mitra, 2020). The Stern-Volmer quenching constant (K_{SV} /10^3 M^{-1}) varies in the range of 3.21 ~ 5.85 and 6.76 ~ 14.26 for the interaction of CPM with BSA and BSA – AuNP conjugate, respectively within the temperature range of 298 ~ 318 K. Interestingly, the association constant (K_a) of the protein – drug complex formation remains almost similar for CPM interaction with native BSA ($3.48\pm0.24\cdot10^3$ M^{-1}) and BSA – AuNP bioconjugate ($3.50\pm0.43\cdot10^3$ M^{-1}) at 298 K. This apparently insignificant change in binding efficiency of CPM with BSA and its nanoconjugate is consistent with negligible change in protein secondary structure as confirmed from far-UV circular dichroism (CD) absorption measurement at 208 nm and by monitoring the position of amide I peak in infra-red (IR) spectra. The experimental results confirm that nanoparticle (AuNP) formulation for the delivery of CPM would enhance its efficacy toward the treatment of DM through

inhibiting AGE product formation without compromising the drug carrying ability of serum protein.

5.1.3. Effect of AuNP on the enzymatic activity of BSA. In contrast to many of its counterpart plasma proteins, serum albumin does not contain a carbohydrate moiety on its surface which gives it greater flexibility in performing its numerous biological functions (Bteich, 2019). Albumin is not just a passive but an active participant in several pharmaco- or toxicokinetic processes (SUDLOW *et al*., 1976) The interaction of serum albumins with esters have gained considerable interest in recent times (Wu *et al*., 2016) In numerous experiments, esterase or pseudo-esterase activity of albumin was demonstrated with respect to α- and β-naphthyl acetate or p-nitrophenylacetate (Sakurai et al., 2004). In this study, the enzymatic activity of BSA has been checked spectrophotometrically in presence of p-nitrophenyl acetate (pNA), by monitoring the quantity of pNA released at 410 nm following the protocol mentioned elsewhere (Østdal & Andersen, 1996). The intrinsic absorbance of AuNP was subtracted for obtaining the corrected optical density. The activity of native BSA is taken to be 100%. The interaction of BSA with the two working concentrations of AuNP in the present experiment (i.e. 0.13 and 0.68 nM) results in only about 6.2±0.3 and 10.8±0.2% loss in enzymatic activity relative to native BSA (Figure 2b, inset). This result implies that addition of AuNP, even at the highest working concentration in this study, does not significantly alter the enzymatic activity of serum protein and therefore, gives a viable option in the field of nanomedicine.

5.2. Effect of AuNP on the AChE activity: Comparing with FDA approved AD drug

5.2.1. Quantification of AChE inhibition. Inhibition of AChE activity is quantified in the presence of FDA-approved reversible inhibitor donepezil hydrochloride monohydrate (DON) following modified Ellman method along with kinetic scheme based on MM mechanism. The kinetic parameters for enzyme hydrolysis ([AChE] = 0.079 u/ml) in 0.1 M phosphate buffer medium of pH = 8 are listed in table 1. The results indicate that while the magnitude of MM constant (K_m) remains constant, the inhibition is associated with the reduction of the V_{max} value, suggesting a non-competitive type of inhibition mechanism to be operative in this case. This is a special type of the mixed inhibition mechanism and involves both the competitive [binding of inhibitor (I) with the enzyme (E) to give EI complex in lieu of the substrate (S) to give ES] and uncompetitive (inhibitor binds with ES to give IES complex) inhibition pathways. This mechanism is further ensured from the equivalence of measured α (=1+[EI]/[I]) and α′ (=1+[IES]/[ES]) values. The calculated IC50 value for the enzyme hydrolysis data is found to be 68.3±3.5 nM.

Table 1. Kinetic data of enzyme hydrolysis and the effect of different concentrations of DON on various kinetic parameters in aqueous buffer and in presence of 250 μM HSA.

[DON] /nM	K_m /μM	V_{max} /nMs^{-1}	α	α′
Aqueous buffer of pH =8 (IC_{50} =68 ± 4 nM)				
0	120 ± 20	815 ± 46	1.0 ± 0.3	1.0 ± 0.1
5	100 ± 10	565 ± 29	1.2 ± 0.3	1.4 ± 0.1
10	120 ± 20	390 ± 26	2.1 ± 0.5	2.1 ± 0.2
250 μM HSA matrix (IC_{50} =159 ± 8 nM)				
0	120 ± 20	872 ± 72	1.0 ± 0.3	1.0 ± 0.1
5	150 ± 20	616 ± 28	1.8 ± 0.4	1.4 ± 0.1
10	140 ± 30	606 ± 35	1.7 ± 0.5	1.4 ± 0.1

Considering the severity of the increased number of affected patients and alarming death statistics in AD, there is a lot of research on the development of new drugs with enhanced therapeutic effectiveness at present. Out of the only five chemical species cleared by FDA towards the treatment of AD till date, pharmaco-kinetic parameters confirm the efficiency of DON to be among the best. Therefore, it is often the usual practice to compare the effectiveness of a newly synthesized drug in buffer solution with that of DON before declaring it a potent pro-drug and subsequent animal trial. Interestingly however, while the ADME of DON is well-known, the effect of HSA on the kinetics of AChE inhibition is not studied in detail. Considering this, an in-vitro investigation is done to monitor the AChE inhibition under the condition of fixed HSA concentration of ca. 250 μM, which is close to the reported level of physiological abundance of serum albumins. The experimental results (table 1) reveal that while the mechanism of AChE inhibition remains the same even in HSA medium, the inhibitory potential decreases significantly with calculated IC_{50} = 158.8±7.9 nm. The extent of this modulation, parametrized by the relative change in IC_{50} value, is compared with similar experimental results for several other AD drugs and is found to be strongly correlated with the sequestration ability of HSA toward the inhibitor drug (Islam *et al*., 2016). While our results point toward a significant question mark on "potential" AChE inhibitors in buffer reported randomly in current literature, it also confirms that drug activity as AChE inhibitor can be strongly modulated depending on the experimental medium.

5.2.2. Effect of AuNPs on enzymatic activity of AChE. Numerous reports showing a considerable effect of NPs on biochemical activity of different enzymes propelled us to check the influence of AuNPs on the enzymatic activity and kinetics of AChE. It is to be noted here that all the nanoparticle experiments are conducted in aqueous buffer medium in these preliminary experiments, since the presence of serum protein (HSA) would render additional complications in standardizing the enzymatic reaction due to the formation of protein-corona. Nevertheless, analysis of the kinetic parameters and the MM plots obtained from the enzyme hydrolysis data (Figure 3) revealed that in case of AuNP, only V_{max} shows a sharp increase. An initial value of 801 nM s^{-1} for unadulterated AChE rises to 1052 nM s^{-1} in presence of 10 nM AuNP. However, the values of K_m for AChE and 10 nM AuNP-treated AChE are 170 ±13 μM and 183 ± 11 μM , implying a practically constant value. Since V_{max} is the rate of reaction when the enzyme is saturated with substrate i.e. the maximum achievable reaction velocity, it can be inferred that AuNPs increase the activity of AChE by enhancing the rate of enzymatic reaction. However, since the magnitude of K_m (which is an inverse measure of the enzyme affinity to the substrate) remains unchanged, it is clear that AuNPs do not cause any alteration in the enzyme-substrate affinity.

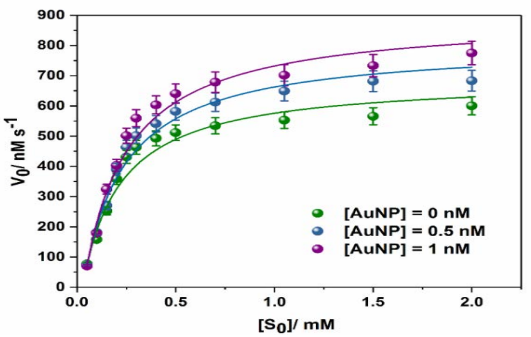

Figure 3. Hydrolysis curve (scattered points) for AChE activity and its inhibition in presence of different concentrations of AuNP in phosphate buffer solution of pH = 8.0. The solid line represents non-linear regression of the experimental data points. [AChE] = 0.079 u/ml.

Comparison of the calculated kinetic parameters indicates that AuNPs act as enzyme activators for AChE, possibly through immobilization on enzyme surface. Typically, enzyme immobilization refers to the optimization of the operational performance of an enzyme by adsorbing it on a matrix of choice. NPs act as very efficient support materials for enzyme immobilization, because of their extreme bio-compatibility. Enzyme bound NPs have been found to show Brownian movement, when dispersed in aqueous solutions, showing the enzymatic activities to be comparatively better than that of the unbound enzyme (Lee & Au-Duong, 2018; Breger *et al*., 2019) Various reviews on immobilization of enzymes on different types of nanoparticles (for example, metal and metal-oxide nanoparticles, magnetic nanoparticles, porous and polymeric nanoparticles etc.) have been reported (Gupta *et al*., 2011; Ansari & Husain, 2012; Verma *et al*., 2013).

In this case too, the activity of AChE is seen to increase in the presence of AuNPs, leading to the conclusion that the enzyme adsorbs itself efficiently on the AuNP surface, leading to a boost in performance.

However, the boosting of AChE activity renders an event of possible toxicity in the purview of Alzheimer's disease (AD) in relation to the cholinergic hypothesis. This is because the higher activity of AChE results in more acetylcholine (ACh) break-down into acetate and choline, a reaction which AChsE catalyses very efficiently. Since a decline in ACh level has been a primary pathological cause of AD, AuNPs (which act as AChE activity boosters) would pose danger in the purview of this deadly disease. Therefore, the unperturbed usage of NPs in neurological functions is restricted despite its advantage of easy BBB permeability due to its small size.

Considered in a wider perspective, the present investigation implies that AuNPs not only put someone who is exposed to suspended NPs through air and/or industrial pollution at risk of incurring AD, but also poses a significant threat towards the effective treatment of the AD-affected population. Proper healthcare formalities might need to be prescribed for patients undergoing treatment for dementia; otherwise, excess use of cholinergic drugs (to achieve the desired result of overcoming the influence of nanotoxicity) may result in drug overdose and additional side effects. This indicates that tweaking the formulations of metallic NPs for usage in medicinal fields, as well as countering the neurologically toxic effects of NPs in people exposed to these for prolonged periods, needs to be approached judiciously

6. Concluding remarks

Nanoparticles have been proven to be extremely valuable in biomedical applications with respect to chemical sensing, bacteria annihilation, biological imaging, drug delivery, and cancer treatment. However, despite all the positive build-up that they have garnered over the years, their effects are not unequivocally beneficial. In this study, we have explored two margins of AuNP with contradictory relevance in the field of medicine.

AuNPs act as potent inhibitors in the glycation of BSA by L-arabinose, demonstrating their usefulness in the treatment of diabetic patients. The anti-glycation properties of AuNPs, followed through their drug binding technique, could be a useful tool in the field of nanomedicine. This result is extremely important because an anti-diabetic drug, CPM, was found to enhance protein glycation, exhibiting a behaviour contrary to what is expected of it. Hence, this might open new avenues, and showcase the necessity of thoroughly examining the pathology of DM, the mechanism of glycation, and their relationship with each other. On the other hand, AuNPs exert a boosting influence on the enzyme kinetics of AChE, implying their toxicity in relation to AD.

Since NP mediated drug delivery is gaining popularity in pharmacology, our

current observations provide new insights, and demand justification of their usage. It further indicates that the haste in using NPs as biological carriers should be accompanied by caution and a thorough understanding of its probable influences on different biological media. Only with exhaustive and strategic approaches can NPs be utilized to their full potential to yield fruitful results in the pharmacological milieu.

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