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Ispitivanje *in vitro* interakcija jedinjenja zlata sa Na^+/K^+ ATPazom

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Investigation of *in vitro* interactions of gold compounds with Na^+/K^+ ATPase

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Naziv: Ispitivanje *in vitro* interakcija jedinjenja zlata sa Na^+/K^+ ATPazom

Izvod:

Na^+/K^+ ATPaza je membranski enzim koji koristi energiju oslobođenu iz molekula adenozin trifosfata da bi regulisao odnos koncentracije jona kalijuma i natrijuma sa obe strane ćelijske membrane većine živih bića. Pored transporterske uloge, on obavlja i funkciju signalnog molekula, jer je uključen u regulaciju aktivnosti Src kinaze, koja je dalje uključena u signalne puteve unutar ćelije i reguliše preživljavanje ćelije. Zbog ovoih svojstava Na^+/K^+ ATPaza je prepoznata kao važna terapeutska meta u velikom broju različitih patogenih stanja uključujući i tumore. Kompleksi plemenitih metala, a naročito zlata predstavljaju novu veliku grupu jedinjenja koja pokazuje sposobnost inhibicije Na^+/K^+ ATPaze. Ovo ih čini potencijalnim terapeuticima, analognim kompleksima platine koji su u sastavu modernih hemoterapija. Osim kompleksnih jedinjenja zlata, interesantna svojstva pokazuju i nanočestice zlata. U ovoj studiji ispitivani su efekti 9 kompleksnih jedinjenja zlata i 3 tipa koloidnih nanočestica zlata na različite preparate enzima Na^+/K^+ ATPaze.

Dobijeni rezultati ukazuju na to da 6 od 9 ispitivanih kompleksa ima umereno jak inhibitorni uticaj na Na^+/K^+ ATPazu. Pored toga, pokazano je da je reč o nekompetitivnoj reverzibilnoj inhibiciji, koja se može otkloniti dodatkom donora –SH grupe kao što su cistein i glutation. Osim toga, inhibicija se može i u potpunosti predupreditи istim ovim supstancijama. Stoga je zaključeno da se vezivanje kompleksa za enzim odvija preko –SH ostataka cisteina u enzimu. Pokazano je da neki od ispitivanih kompleksa imaju izuzetno jak citostatski efekat, što ih čini dobrim kandidatima za dalje medicinske studije. Upotrebom MALDI TOF masene spektrometrije pokazano je da postoji direktna reakcija između kompleksa i donora –SH grupe, tj da ove dve vrste direktno reaguju kada su prisutne u rastvoru.

Nanočestice zlata koje su ispitivane nisu dale zapažen inhibitorni efekat. Tip nanočestica obeležen kao C3 koji sadrži nanočestice zlata prečnika 9,5 nm, pokazao je neobična svojstva - dodatkom u preparat sinaptozomalnih plazma membrana, doveo je do porasta aktivnosti Na^+/K^+ ATPaze u tom preparatu dvostruko, dok se aktivnost ostalih prisutnih enzima nije menjala. Ispitivanjem ove interakcije pokazano je da je u pitanju fizička promena u preparatu do koje dolazi usled dva tipa hemijske interakcije između koloida i preparata enzima. Uočene su promene u UV-vis i FTIR spektrima koloida po dodatku enzima, a upotrebom atomske mikroskopije uočeno je da dolazi do promena u nanostrukturi preparata enzima koje izaziva povećanje aktivne površine ćelijske membrane, te tako veći broj molekula enzima postaje dostupan za reakciju i javlja se porast aktivnosti enzima u preparatu. Selektivnost enzima za njegove prirodne ligande ostaje neizmenjena, a dobijeni preparat pojačane aktivnosti stabilan je najmanje sat vremena.

Zaključeno je da ispitivani kompleksi zlata mogu da budu od značaja za dalji razvoj antitumorskih terapija, kao i da koloidne nanočestice zlata mogu poslužiti za razvoj osetljivijih testova za merenje aktivnosti membranskih enzima.

Ključne reči: Na^+/K^+ ATPaza, kompleksi, zlato, nanočestice, inhibicija, reaktivacija, sprečavanje, koloid, citotoksičnost, imobilizacija.

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Abstract

Na^+/K^+ ATPase is a membrane enzyme that uses the enegry released from the molecule of adenosine triphosphate to regulate the sodium and potasium ion concentration on either side of the cell membrane in most living creatures. Appart from its transporter role, it also has a role as a signal molecule, because it regulates the activity of Src kinase, an important enzyme in cell signaling and survival. Beacuse of these properties, Na^+/K^+ ATPase is recognized as an important molecular target in many disorders including tumors. Noble metal complexes, and especially gold complexes have shown the tendency to inhibit Na^+/K^+ ATPase. This makes them potential terapeutics, analogous to platinum based complexes that are a part of modern chemotherapy. Colloid nanoparticles of gold have also shown interesting properties in the past. This study presents the findings on the interaction of several preparations of Na^+/K^+ ATPase with 9 different complexes of gold and 3 different types of gold colloid nanoparticles.

Results shown in this thesis indicate that 6 out of 9 investigated complexes have a moderatley strong inhibitory effect upon the Na^+/K^+ ATPase. It is shown that this inhibition is noncompetitive and reversible, and that it can be reversed by –SH group donors such as cysteine and glutathione. Moreover, this inhibition can be fully prevented by adding these substances prior to the addition of the complex. Therefore it is concluded that the binding of these complexes takes place through –SH groups of cysteine residues in the enzyme. Some of the investigated complexes have shown a strong cytotoxic effect upon human lymphocytes, which makes them good candidates for further clinical studies. MALDI TOF mass spectroscopy has shown that there is a direct reaction between –SH group donors and the gold complex.

Investigated nanoparticles yielded no significant inhibitory effect. One type of the nanoparticles, however, has shown some very unusual properties. Upon the addition of this type of colloid nanoparticles, labeled C3 and with an average diameter of 9.5 nm, to the preparation of synaptosomal plasma membranes, the activity of Na^+/K^+ ATPase doubled, while the activity of other present enzymes remained the same. Further investigation of this interaction has shown that the cause of this is a physical change in the structure of the membrane fragments. It is the result of two paralel chemical interactions that take place when the nanoparticles are added to the enzyme preparation. Changes in UV/vis and FTIR spectra of the nanoparticles were observed and using atomic force microscopy it was possible to visualize the change in the nanostructure of the enzyme preparation upon the addition of nanoparticles. This change resulted in an increase of the active membrane surface, exposing more molecules of the enzyme and making them available for the reaction. This caused an increase of the enzyme activity. Selectivity of the Na^+/K^+ ATPase towards its natural ligands was preserved, and the effect of increased activity persisted for at least an hour before diminishing.

In conclusion, investigated complexes and nanoparticles of gold have shown important properties. The effects of gold complexes make them potential antitumor drugs, while the nanoparticles of gold may be used to develop better assays for membrane enzymes.

Key words: Na^+/K^+ ATPase, complex, gold, nanoparticle, inhibition, reactivation, prevention, colloid, cytotoxicity, immobilisation.

Scientific field: Life sciences

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Spisak skraćenica

ATP – Adenozin trifosfat

EC – Enyzme commision, broj koji je Enzimska komisija dodelila nekom enzimu

Na^+/K^+ ATPaza – Natrijum kalijum zavisna adenozin trifosfataza

ADP – Adenozin difosfat

AMP – Adenozin monofosfat

cAMP – Ciklični adenozin monofosfat

MAPK – Mitogenom aktivirana protein kinaza

NCX – Na^+-Ca^{2+} eXchange, sistem za antiport jona kalcijuma i natrijuma.

DMSO – Dimetil sulfoksid

DNK – Dezoksiribonukleinska kiselina

EDTA – Etilen diamin tetraacetatna kiselina

TrisHCl – Tris(hidroksimetil)aminometan

FTIR – Infracrvena spektroskopija obrađena Furijeovom transformacijom

ATR – Zadržana (umanjena) totalna refleksija pri snimanju FTIR spektra

AFM – Atomska mikroskopija

HOPG – Visokoorijentisani pirolitički grafit

IC_n – Koncentracija inhibitora koja dovodi do n% inhibicije

GSH – Redukovani glutation

K_M – Mihelis-Mentenina konstanta

v_{max} – Maksimalna teorijska brzina enzimske reakcije

K_i – Konstanta inhibicije

MALDI – Laserska desorpcija i jonizacija potpomognuta matriksom

TOF MS – Masena spektrometrija u kojoj se meri vreme leta jona

DHB – 2,5 – dihidroksibenzoeva kiselina

Sadržaj

1. Uvod

Na^+/K^+ zavisna adenozin trifosfataza (Na^+/K^+ ATPaza) (EC 3.6.3.9.) je enzim i antiporterski sistem koji obavlja izuzetno važnu ulogu aktivnog transporta jona natrijuma i kalijuma kroz ćelijsku membranu životinja. Uz utrošak energije koja nastaje hidrolizom molekula adenozin trifosfata, ovaj enzim izbacuje jone natrijuma iz ćelije i ubacuje jone kalijuma u ćeliju. Kako je koncentracija jona sa obe strane ćelijske membrane bitan parameter za funkciju ćelije, normalna funkcija Na^+/K^+ ATPaze je od vitalnog značaja za ćeliju. Upravo njena važna uloga u ćelijskom metabolizmu čini Na^+/K^+ ATPazu atraktivnom molekulskom metom za razvoj farmaceutika koji bi mogli na različite načine da modifikuju ponašanje ćelija. Najstariji fiziološki aktivni molekuli koji su u medicinskoj upotrebi, a koji svoje fiziološko dejstvo ispoljavaju preko inhibicije Na^+/K^+ ATPaze su kardiotonični glikozidi iz lekovitog bilja koji se već vekovima koriste u terapiji kardiovaskularnih oboljenja [1].

Skorašnje studije pokazale su da je Na^+/K^+ ATPaza jedna od meta antitumorskih lekova na bazi kompleksa plemenitih metala koji vode poreklo od cisplatina, antitumorskog leka koji je u upotrebi od 1978. godine kao deo hemoterapije malignih tumora [2-4]. Veliki uspeh cisplatina usmerio je potragu za antitumorskim lekovima prema kompleksima drugih plemenitih metala [5-7]. Jedna od obećavajućih grupa jedinjenja su i kompleksi zlata Au(III) [8]. U savremenoj literaturi velika pažnja je posvećena upravo interakciji ovih kompleksa sa njihovim metama na molekulskom nivou i razjašnjavanju procesa koji se tu odvijaju. Zlato zavređuje naročitu pažnju zbog velikog afiniteta za vezivanje sa sumporom koji je prisutan u proteinima, a izostaje iz nukleinskih kiselina. U poslednjih nekoliko godina je sintetizovan veliki broj novih kompleksa zlata i svakodnevno se pojavljuju novi rezultati u ovoj oblasti, što je jasan indikator da je ovo polje hemije vrlo aktuelno u svetskoj naučnoj javnosti. Jedan od mehanizama delovanja ovih jedinjenja koji je značajno proučiti jeste inhibicija Na^+/K^+ ATPaze, jer kao što je navedeno analogni kompleksi platine

inhibiraju ovaj enzim, za koji je nedavno pokazano da je vrlo direktno uključen u procese preživljavanja ćelije [9, 10]. Osim inhibicije, važno je proučiti i interakcije potencijalnih antitumorskih lekova sa drugim molekulima u organizmu koji bi mogli umanjiti njihovu terapijsku vrednost. U slučaju zlata to su molekuli koji nose –SH grupu, poput cisteina, glutationa i proteina prisutnih u krvi. Takođe je važno razmotriti mogućnosti uklanjanja inhibicije, ukoliko dođe do neželjenih dejstava. Sa strukturne strane, poželjno je ispitati što detaljnije koji motivi proteinske strukture su uključeni u vezivanje sa kompleksom i šta se događa sa kompleksom nakon što obavi svoju funkciju. Za sada je opisano nekoliko fenomena koji se događaju po vezivanju zlata iz kompleksa za biomolekule koji sadrže sumpor, od kojih je najznačajnija redukcija zlata Au(III) u zlato Au(I) i Au^0. Od značaja za studiju ovog tipa je uvid koji se može dobiti primenom metoda strukturne i mikroskopske analize, kao što su tehnike masene spektrometrije, infracrvene i UV i vidljive spektroskopije, kao i tehnika atomske mikroskopije za posmatranje strukturnih promena materijala na nano nivou.

Kako je redukcija zlata u vodenim sredinama do elementarnog zlata često praćena formiranjem nanočestica zlata, postavlja se pitanje interakcije nanočestica, koje su već pokazale biološke efekte na bakterijskim i ćelijskim kulturama, na funkciju Na^+/K^+ ATPaze [11]. U literaturi, međutim, ne postoji dovoljno podataka koji se bave ovom interakcijom. Podaci o interakciji Na^+/K^+ ATPaze sa kompleksima zlata takođe su vrlo ograničeni, te se stoga ovde otvara prostor za istraživanje.

Najzad, kako sama interakcija na molekulskom nivou ne mora imati reperkusije na funkciju ćelije u celosti, postavlja se i pitanje biološkog efekta ovih jedinjenja na modele humanih ćelija. U literaturi su opisani efekti do sada poznatih jedinjenja iz ove grupe [12]. Oni obuhvataju sprečavanje ćelijske proliferacije, remećenje procesa koji su od vitalnog značaja kao što su replikacija DNK, sinteza proteina i primanje signala faktora preživljavanja. Iako mehanizmi nisu u potpunosti poznati, očekivano je da će nova kompleksna jedinjenja zlata proizvesti iste efekte. Neka od ispitivanih jedinjenja već su pokazala citotoksične efekte na ćelijama raka, ali nisu ispitivani njihovi mehanizmi dejstva, kao ni njihovi efekti na zdrave ćelije.

Sva ova pitanja poslužila su kao motiv za sprovođenje ove studije. Neka od ovih pitanja su dobila odgovor tokom ove studije i poslužiće kao polazna tačka za dalje analiziranje ove problematike.

2. Opšti deo

2.1. Struktura ćelijske membrane

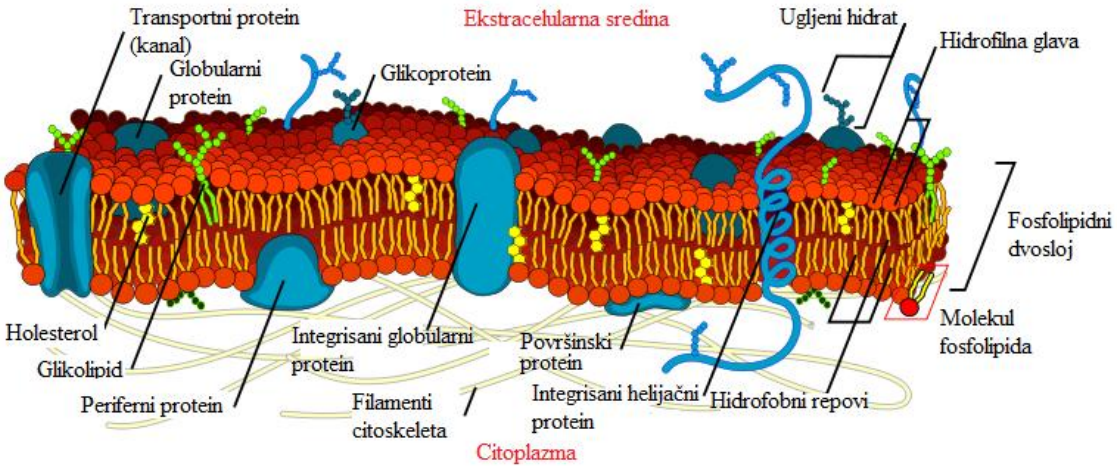
Svaka ćelija živog sveta oivičena je ćelijskom membranom. Ćelijska membrana predstavlja funkcionalnu, anatomsku i fizičku barijeru koja odvaja ćeliju od okolne sredine [13]. Ipak, kako ćelija svoj kompletan metabolizam obavlja uz interakciju sa okolinom, ćelijska membrana ujedno i spaja ćeliju sa okolnom sredinom i drugim ćelijama. U sastav ćelijske membrane ulaze fosfolipidi koji formiraju fosfolipidni dvosloj, glavni gradivni materijal ćelijske membrane. Fosfolipidi su jednim svojim krajem hidrofilni, orijentisani ka spoljašnjoj sredini, a drugim krajem su hidrofobni i u dodiru sa hidrofobnim krajevima obrnuto orijentisanih fosfolipida, koji su svojim hidrofilnim krajevima orijentisani prema unutrašnjosti ćelije. Ovako formiran hidrofobni "sendvič" sprečava slobodno cirkulisanje hidrofilnih supstanci u ćeliju [14, 15].

Osim fosfolipida u fosfolipidnom dvosloju zastupljeni su u manjoj meri i u zavisnosti od vrste kojoj ćelija pripada i sfingolipidi, liposaharidi, steroidni molekuli, etri, voštane materije i mnogi drugi lipofilni molekuli koji formiraju lipidnu barijeru [14]. Ipak, kako ćelija mora komunicirati sa okolinom, u membrani postoje uronjeni i proteini koji obavljaju bilo transportnu, bilo signalnu ulogu. Ovi proteini, ukoliko im je uloga transportna, vezuju određene molekulske vrste s jedne strane membrane i nakon konformacionih promena prebacuju ih sa druge strane membrane. Transport kroz membranu može se odvijati uz utrošak energije ili pak bez utroška energije, u zavisnosti od toga da li je termodinamički povoljan transport date materije kroz membranu. Ukoliko se za transport troši energija, ona se uglavnom obezbeđuje iz visokoenergetskih molekula koji su prisutni u ćeliji, poput adenozin trifosfata (ATP). Ukoliko je transport pasivan i ne troši energiju, postoji mogućnost da ćelija na račun tog transporta proizvede energiju ili da se on pak odvija bez nastanka energije [16].

Osim transportnih proteina, postoje i pomenuti signalni proteini tj. receptori. Oni ne transportuju materije kroz membranu, ali transportuju informacije kroz membranu. Po vezivanju neke signalne molekulske vrste sa jedne strane membrane za sebe, oni doživljavaju strukturne i funkcionalne promene koje za posledicu imaju pokretanje hemijskih događaja sa druge strane membrane. Time se bez direktnog prisustva signalnog molekula unutar ćelije događaju hemijske reakcije koje regulišu metabolizam ćelije i odlučuju o daljim događajima u njoj [17, 18].

Membrana ćelije nije čvrsta struktura. Ona je u stalnom kretanju i u tečnoj konzistenciji. Često se opisuje kao "kap ulja na površini supe". Ovakav teorijski model membrane naziva se tečno mozaični model membrane i predstavlja aktuelno prihvaćen model. Oblik ćeliji daju elementi ćelijskog skeleta koji su pričvršćeni za ćelijsku membranu sa unutrašnje strane membrane. Bez njih, ćelija bi zauzela kapljičast sferan oblik. Osim proteina koji su za ćelijsku membranu vezani sa unutrašnje strane, kod višećelijskih organizama ćelije su povezane sa okolnim ćelijama i sa spoljašnje strane. Kod ćelija koje tokom svoje normalne funkcije menjaju oblik upravo elementi citoskeleta utiču na taj oblik. S druge strane kod ćelija koje su oivičene ćelijskim zidom, oblik diktira ćelijski zid [14, 15].

Ćelijske membrane u svom sastavu često imaju enzime. Ponekad su ovi enzimi uronjeni u membranu, a ponekad su samo priključeni membrani sa spoljašnje ili unutrašnje strane. Pojedini membranski proteinski kompleksi obavljaju više funkcija pa mogu biti u isto vreme i enzimi i signalni molekuli i transporteri manjih molekula [19].



Slika 1. – Šematski prikaz ćelijske membrane.

2.2. Podela i osnovne karakteristike enzima

Enzimi su proteinski biokatalizatori koji se nalaze u svim živim bićima i katalizuju odvijanje biohemijskih reakcija koje omogućuju život kao proces. Bezbedno je reći da bez enzima ne postoji ni život na našoj planeti. Svaki enzim ima svoj supstrat – molekul ili molekule nad kojima vrši svoju katalitičku ulogu i za koje je u manjoj ili većoj meri selektivan [20]. Svi enzimi se dele prema reakcijama koje katalizuju: oksidoreduktaze (EC broj 1, katalizuju reakcije oksidacije i redukcije atomskih ili molekulskih vrsta), transferaze (EC broj 2, katalizuju transfer atoma ili atomskih grupa sa jednog molekula na drugi), hidrolaze (EC broj 3, katalizuju hidrolizu hemijskih veza, uvođenjem molekula vode), lijaze (EC broj 4, katalizuju raskidanje hemijske veze bez oksidacije ili utroška vode), izomeraze (EC broj 5, katalizuju prelazak jednog izomera nekog molekula u drugi) i ligaze (EC broj 6, katalizuju formiranje kovalentnih veza između molekula.

Svaki enzim ima svoj aktivni centar, koji predstavlja region enzimske strukture najneposrednije uključen u proces prepoznavanja i vezivanja supstrata, kao i u proces

hemijske katalize koju taj enzim vrši. Enzim može imati više aktivnih centara [21]. Pored aktivnog centra enzim može imati i regulatorna mesta drugde u svojoj strukturi, koja služe za vezivanje atomskih ili molekulskih vrsta koje povećavaju ili smanjuju aktivnost enzima u skladu sa trenutnim potrebama organizma. Enzimi pored supstrata mogu vezivati različite koenzime i kofaktore, molekulske ili atomske vrste neophodne za funkciju enzima, ali iz enzimske reakcije izlaze nepromenjeni [22]. Svaki enzim karakterišu njegovi funkcionalni parametri, poput pH i temperaturnog optimuma, koji predstavljaju pH vrednost, odnosno temperaturu na kojoj enzim najbolje obavlja svoju katalitičku ulogu.

Pored ovih, postoje i veoma važni kinetički parametri, kao što su K_M (Mihelis-Mentenina konstanta, koja predstavlja konstantu stabilnosti kompleksa enzim-supstrat) i v_{max} (maksimalna teorijska brzina enzimske reakcije), koji figurišu u Mihelis-Menteninoj jednačini enzimske kinetike i predstavljaju važne parametre prilikom razmatranja ponašanja enzima u različitim uslovima i u prisustvu inhibitora ili aktivatora. Mihelis-Mentenina jednačina glasi:

$$v_0 = \frac{V_{\mathbf{max}}[\mathrm{S}]}{K_M + [\mathrm{S}]}$$

gde je v_0 brzina reakcije, [S] koncentracija supstrata, K_M Mihelis-Mentenina konstanta, a v_{max} maksimalna brzina enzimske reakcije.

Enzimi mogu biti inhibirani (sprečeni da vrše enzimsku funkciju) pomoću molekula koji se nazivaju inhibitori. Inhibitori mogu biti reverzibilni ili ireverzibilni. Kod ireverzibilnih inhibitora enzimska funkcija je trajno onesposobljena čvrstim, najčešće kovalentnim vezivanjem inhibitora za enzim. Reverzibilna inhibicija ima nekoliko podtipova koji se razlikuju prema tome koji kinetički parametar enzima menjaju: kompetitivna (u kojoj inhibitor kompetira za vezivanje u aktivno mesto sa supstratom i gde se smanjuje K_M, a v_{max} se ne menja), akompetitivna (u kojoj inhibitor vezuje formirani enzim-supstrat kompleks i sprečava dalju reakciju i gde se i K_M i v_{max} smanjuju za isti faktor i gde postoji jedna konstanta inhibicije), mešovitu (u kojoj se i supstrat i inhibitor vezuju za enzim, jedan utičući na vezivanje drugoga i gde se i K_M i v_{max} menjaju ali za različite faktore i gde

postoje dve konstante inhibicije) i nekompetitivnu (u kojoj se inhibitor vezuje nezavisno od supstrata i gde se K_M ne menja, ali se v_{max} smanjuje). Osim ovih tipova inhibicije postoji i specijalni slučaj koji se naziva inhibicija supstratom [23].

2.3. Podela i uloge adenozin trifosfataza

Adenozin trifosfataze (ATPaze) su raznorodna grupa enzima iz familije hidrolaza, koji spadaju u grupu hidrolaza koje deluju na anhidride kiselina, tačnije u EC grupe 3.6.3 (transportne) i 3.6.4. (strukturne) . Ono što je ključna karakteristika enzima ove grupe jeste njihova sposobnost da koriste hemijsku energiju skladištenu u molekulu ATP koji hidrolizuju i da tako dobijenu energiju koriste za obavljanje svojih funkcija. Najčešći vid hidrolize ATP je njegovo razlaganje na jon fosfata i molekul adenozin difosfata (ADP), ali je takođe poznat i slučaj u kojem se oslobađa jon pirofosfata i molekul adenozin monofosfata (AMP), na primer tokom sinteze nukleinskih kiselina. Pošto ATP i srodni niži fosfati adenozina nose veliko negativno naelektrisanje, najčešće se u reakcijama koje katalizuju ATPaze javlja jon magnezijuma. ATPaze se mogu podeliti u nekoliko familija na osnovu njihove osnovne biohemijske uloge.

- F-ATPaze (fosforilacione ATPaze) su uključene u sintezu ATPa i izuzetno su konzervirane kroz evoluciju. Najčešće su selektivne za H^+ jone, ali u pojedinim bakterijama postoje i one koje su Na^+ selektivne. Lokalizovane su u membranama bakterija, tilakoidima hloroplasta i unutrašnjim membranama mitohondrija [24].
- P-ATPaze (ili E1-E2 ATPaze) su jonske pumpe koje se nalaze u velikom broju organizama i postoje razni tipovi ovih enzima. Postoje one koje su selektivne za razne zemnoalkalne i alkalne metale, ali kod mikroorganizama mogu biti selektivne i za teške i toksične metale poput žive, olova ili kadmijuma. Nazivaju se E1-E2 zato što tokom funkcionisanja zauzimaju dve konformacije [25].
- V-ATPaze su vakuolarne ATPaze ali se uprkos nazivu nalaze i u membranama Goldži aparata, endoplazmatskog retikuluma, lizozoma i tako dalje. Njihova uloga

je pre svega pumpanje H^+ jona u ove membranske odeljke radi zakišeljavanja sredine [24, 26].

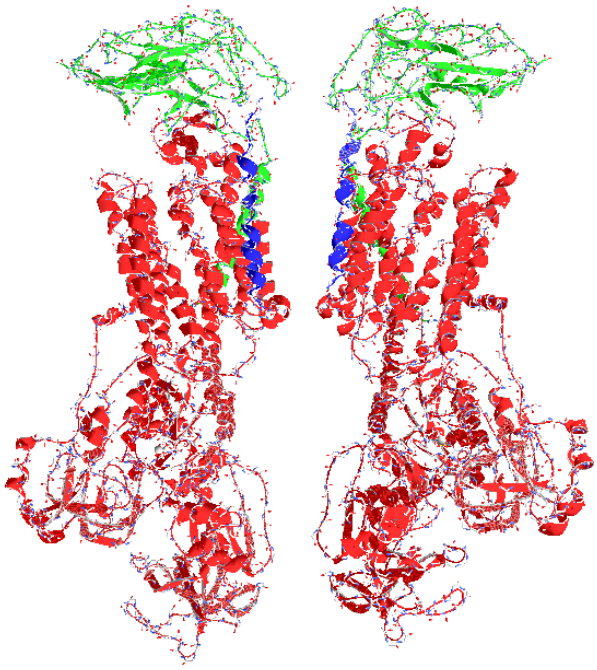
- A-ATPaze su ATPaze koje se nalaze isključivo u organizmima iz carstva *Archea*. Imaju sličnu ulogu kao F-ATPaze, ali su strukturno sličnije V-ATPazama. Pretpostavlja se da su nastale kao evolutivni odgovor na uslove visoke kiselosti sredine u kojima pojedine arhee žive [27].
- E-ATPaze su ekstracelularne ATPaze. To su solublini enzimi koji imaju veoma široku supstratnu specifičnost (nisu selektivne samo za adenozin trifosfat, već i za druge nukleozid trifosfate) [28].

Posebno je značajna grupa EC 3.6.3. koju čine P-ATPaze uključene u aktivni transport molekulskih i atomskih vrsta, jer one obavljaju važan zadatak transporta koji je suprotan koncentracionom gradijentu i samim tim troši značajnu količinu energije. Ovo je naročito izraženo kod ATPaza koje utiču na koncentracije jona alkalnih, zemnoalkalnih metala i vodonika, jer je mineralna i osmotska homeostaza jedan od najvažnijih procesa koji se odvijaju na membranama ćelija svih živih bića. Energetske potrebe ovih enzimskih sistema su ogromne i jedan od prvih znakova energetskog kolapsa ćelije je nemogućnost očuvanja mineralne homeostaze i membranskog potencijala koji upravo obezbeđuju jontransportne ATPaze. Među njima kvantitativno je najzastupljenija i funkcionalno najvažnija ATPaza koja transportuje jone natrijuma i kalijuma, tzv. Na^+/K^+ zavisna adenozin trifosfataza ili "pumpa za natrijum i kalijum" [19].

2.4. Struktura i funkcija $Na^{+/}K^+$ ATPaze

Na^+/K^+ zavisna adenozin trifosfataza (Na^+/K^+ ATPaza, EC 3.6.3.9.) je membranski enzim koji pripada grupi transportnih ATPaza. Ono po čemu je Na^+/K^+ ATPaza specifična jeste njena struktura i uloga u održavanju osmotske i elektrohemijske homeostaze sa obe strane ćelijske membrane životinjskih ćelija. Funkcionalno jezgro enzimskog kompleksa čine dve proteinske podjedinice, α i β podjedinica. U zavisnosti od biološke vrste iz koje je enzim ili

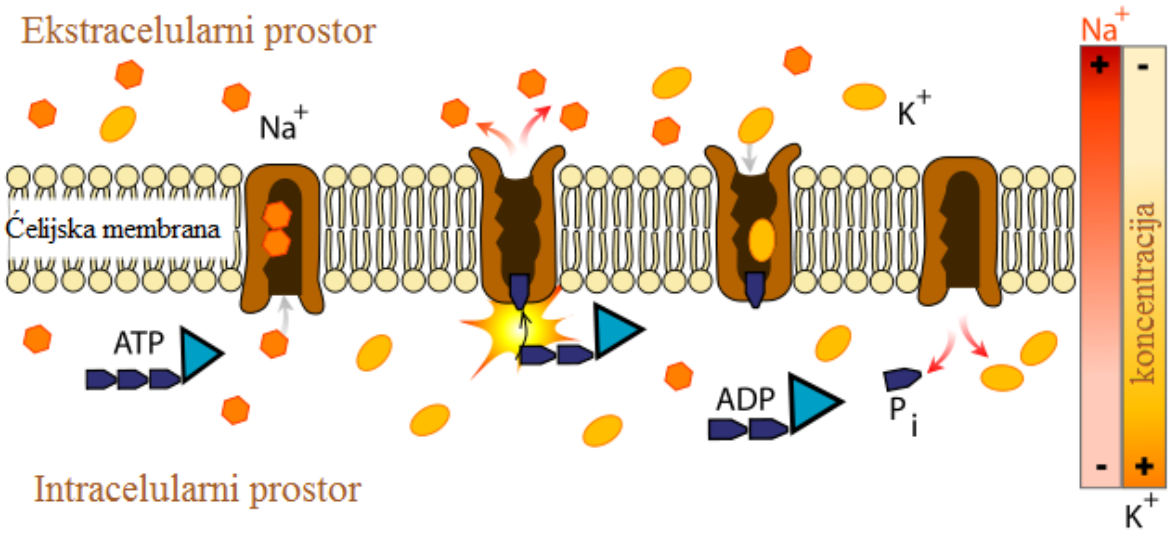
od tipa ćelije u kojoj se nalazi, Na^+/K^+ ATPaza može imati i "priključene" podjedinice i peptide koje nisu od suštinskog značaja za funkciju enzima, već učestvuju u njegovoj regulaciji. U komercijalnim preparatima prisutni su dominantno $\alpha\beta_2$ koji imaju katalitičku funkciju. α podjedinica je mase oko 113 kDa i ona sadrži aktivno mesto gde se hidrolizuje ATP, kao i mesta gde se vezuju joni kalijuma i natrijuma prilikom transporta. Na ovoj podjedinici se nalaze i mesta za vezivanje inhibitora poput kardiotoničnih glikozida. β podjedinica je mase između 50 i 60 kDa i ona je glikoprotein. Neophodna je za normalnu funkciju enzima. Dimenzije enzima su približno 7 × 6 × 15 nm. Tačna topologija enzima je još uvek predmet istraživanja jer postoje kontradiktorni podaci o tačnoj strukturi transmembranskog regiona. U primarnoj strukturi enzima identifikovano je između 58 i 64 cisteinskih ostataka, u zavisnosti od biološkog porekla i izoforme enzima [19, 23, 29-31].



Slika 2. Struktura Na^+/K^+ ATPaze izolovane iz domaće svinje (*Sus scrofa*), α podjedinice su prikazane crvenom bojom, β podjedinice zelenom, prateći stabilizacioni peptid plavom.

Funkcija Na^+/K^+ ATPaze se odvija ciklično. U svakom ciklusu troši se jedan molekul ATP i iz ćelije se izbacuju tri jona natrijuma, a u ćeliju se ubacuju dva jona kalijuma. Ciklus započinje vezivanjem dva jona natrijuma za enzim za koji je već vezan ATP. Potom dolazi

do hidrolize ATP i konformacione promene u enzimu koja dovodi do orijentisanja vezivnih mesta za natrijum prema spoljašnjosti ćelije. Afinitet enzima za natrijum se smanjuje, dok se afinitet prema kalijumu povećava. Po vezivanju kalijumovih jona, zasad nepoznatim mehanizmom, dolazi do defosforilacije enzima i vezivanja novog molekula ATP koji ne hidrolizuje, već se enzim vraća u prvobitnu konformaciju i otpušta jone kalijuma. Tako enzim postaje spreman za novi ciklus [19, 29-31] [23].



Slika 3. Ciklus Na^+/K^+ ATPaze.

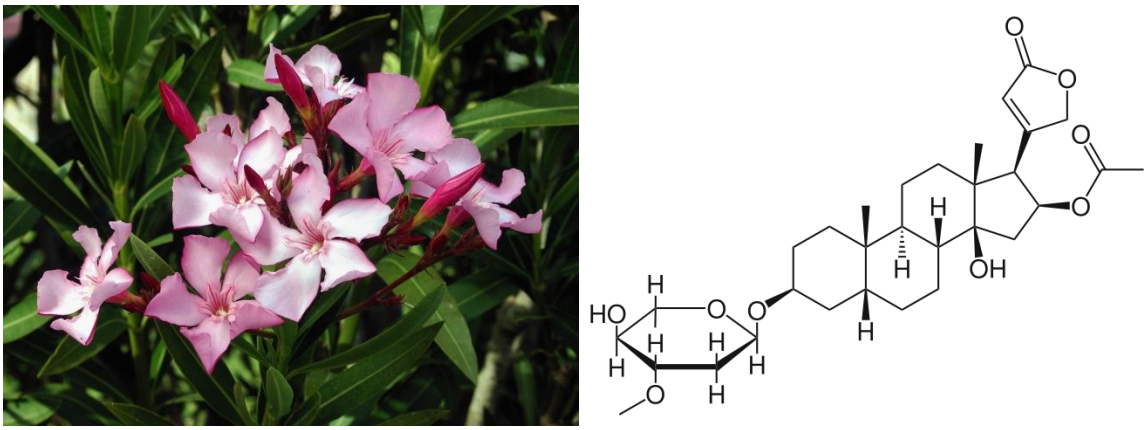
Pored njene uloge u transportu jona, Na^+/K^+ ATPaza ima i važnu ulogu u ćelijskoj signalizaciji. Kako je ona membranski enzim i kako može biti fosforilisana tokom svojeg radnog ciklusa, Na^+/K^+ ATPaza je sposobna da mobiliše molekule poput Src kinaze, nereceptorske tirozin kinaze koja je uključena u signalne kaskade, i da reguliše njihovu aktivnost. U slučanju Src kinaze, Na^+/K^+ ATPaza inhibira Src vezujući je za sebe dok je u fosforilisanom stanju i time reguliše njenu aktivnost. Kada je Na^+/K^+ ATPaza inhibirana kardiotoničnim glikozidima ona ostaje u fosforilisanom stanju, što ima za posledicu i inhibiciju Src kinaze. Pokazano je da Na^+/K^+ ATPaza interaguje i sa ankirinom i fosfoinozitid-3-kinazom. U zavisnosti od anatomske lokalizacije, Na^+/K^+ ATPaza može aktivirati MAP kinazne puteve u ćeliji, fosfolipaza C zavisne kaskade ili inozitol trifosfat zavisne kaskade. Sve ovo čini Na^+/K^+ ATPazu važnim činiocem regulacije u ćelijskoj signalizaciji [9, 10].

Poznato je više endogenih faktora koji direktno ili indirektno regulišu aktivnost Na^+/K^+ ATPaze, ali takođe postoje i nepoznati faktori koji komplikuju razumevanje regulacije ovog enzima. Za sada se zna da ciklični adenozin monofosfat (cAMP) koji je deo sistema sekundarnog glasnika, dovodi do pojačane aktivnosti ovog enzima. Hormoni poput steroidnih polnih hormona i insulina tokom vremena dovode do povećanja brojnosti enzima na membranama ćelija. Insulin ima i akutno delovanje koje dovodi do povećanja afiniteta enzima za vezivanje jona natrijuma i rasta brzine reakcije, dok kateholamini mogu imati suprotna dejstva u različitim tkivima. Važni nedavno otkriveni modulatori aktivnosti ovog enzima su i mali proteini iz FXYD familije, koji su tkivno specifični kratki proteini koji jednom prolaze kroz membranu i vezani su direktno za transmembranske regione α podjedinice (prikazani plavom bojom na slici 2.) [32]. Pored ovoga postoje malobrojni podaci u literaturi o "endogenom uabainu", otkrivenom 1991. godine, steroidnom molekulu najverovatnije poreklom iz nadbubrežne žlezde koji se luči u nanomolarnim ili čak pikomolarnim koncentracijama i ima istovetno dejstvo kao i kardiotonični glikozidi koji inhibiraju enzim [33-36]. Jako fiziološko dejstvo, različiti biološki efekti i ukršteni signalni putevi u regulaciji ovog enzima upućuju na to da je u pitanju veoma delikatan regulatorni sistem, što dalje ukazuje na izuzetnu važnost ovog enzima. Do nedavno je Na^+/K^+ ATPaza smatrana za jednostavan transportni sistem, ali skorašnja istraživanja pokazala su da ona ima veliki značaj u osiguravanju ćelijskog preživljavanja, ne samo kroz osiguravanje osmotske stabilnosti, već i kroz učešće u ćelijskoj signalizaciji. Sposobnost Na^+/K^+ ATPaze da receptuje signale preživljavanja i da utiče na signalne kaskade u citoplazmi je novo otkriće i tek čeka da bude istraženo i shvaćeno u potpunosti [37, 38].

U egzogene faktore koji utiču na funkcionisanje Na^+/K^+ ATPaze spadaju njeni poznati inhibitori.

2.5. Inhibitori Na^+/K^+ ATPaze

Poznat je veliki broj prirodnih i sintetičkih inhibitora Na^+/K^+ ATPaze. Najdužu upotrebu u medicini imaju kardiotonični glikozidi. Kardiotonični glikozidi predstavljaju grupu steroidnih glikozida uglavnom poreklom iz biljaka, uz par izuzetaka koji vode poreklo od životinja. Najpoznatiji su glikozidi iz biljaka roda *Digitalis*, *Stophantus*, *Nerium*, *Helleborus*. [23] Osim ovih, poznat životinjski izvor kardiotoničnih glikozida je rod vodozemaca *Bufo*, odnosno žaba krastača. Kardiotonični glikozidi se prema strukturi dele na kardenolide i bufadienolide. Sastoje se od šećernog (glikonskog) dela i steroidnog (aglikonskog) dela. Aktivni region je steroidni deo koji se vezuje za α podjedinicu Na^+/K^+ ATPaze u fazi kada ona za sebe ima vezan neorganski fosfat i stabilizuje taj enzimski oblik, efektivno sprečavajući dalje normalno obavljanje funkcije ovog enzima. U živoj ćeliji srčanog mišića ovo dovodi do povećanja intracelularnog natrijuma, što za uzvrat dovodi do inhibicije unosa natrijumovih jona kroz NCX kompleks koji je Ca^{2+}/Na^+ antiport. To rezultuje povećanjem unutarćelijskog kalcijuma i prolongira kontrakciju srčanog mišića. Ovaj biološki efekat je od značaja za terapiju kardiovaskularnih bolesti. Ukoliko se pak terapijska doza kardiotoničnih glikozida prekorači, može nastupiti kardiovaskularni kolaps i smrt. Ovaj biološki efekat karakterističan je za jake i selektivne inhibitore Na^+/K^+ ATPaze i ispoljava se samo na ćelijama srčanog mišića zbog specifičnog načina na koji je regulisana njihova kontrakcija [39-41].



Slika 4. Oleander (*Nerium oleander*) i njegov kardiotonični glikozid oleandrin.

Pored kardiotoničnih glikozida, postoje i drugi, manje ili više specifični inhibitori Na^+/K^+ ATPaza. Najpotentniji poznati neglikozidni inhibitor je palitoksin iz nekoliko rodova korala reda *Zoantharia*, koji je ujedno jedan od najjačih nepeptidnih toksina poznatih čoveku. Za razliku od glikozida, Na^+/K^+ ATPazu inhibira u stanju koje dozvoljava pasivni transport jona i time remeti normalan membranski potencijal. Efekti na tkiva se takođe razlikuju od onih koje izazivaju kardiotonični glikozidi [42].

Pored organskih inhibitora, postoji i puno neorganskih vrsta koje su u *in vitro* uslovima pokazale sklonost ka inhibiranju Na^+/K^+ ATPaze. Pre svega to su joni teških metala – olova, žive, kadmijuma, zatim bakra, gvožđa i zlata, naročito zlata Au(I) [43]. Ovi efekti nisu selektivni i posledica su urođenog afiniteta ovih vrsta ka vezivanju za proteine. Pored jona metala volframatni i vanadatni joni su pokazali izuzetnu specifičnost u vezivanju sa Na^+/K^+ ATPazom, usled strukturnih sličnosti sa jonom fosfata. Pojedine vrste ovih jona su čak dalje razvijane kao potencijalni terapeutici. Kompleksni joni plemenitih metala imaju jak inhibitorni uticaj na Na^+/K^+ ATPazu i slične ATPaze i druge enzime. Do sada su u literaturi opisani kompleksi platine, paladijuma i nedavno zlata [3, 44-46].

2.6. Karakteristike kompleksa metala

Kompleksi metala su koordinativna jedinjenja (jedinjenja u kojima je prisutan koordinativni tip veze) u kojima je koordinativni centar jon metala, za koji su koordinativno vezani ligandi. Ligandi u kompleksima metala mogu biti neorganski (malih neorganski molekuli ili joni) ili organski (organski ili bioorganski joni ili molekuli).

Tipični kompleksi metala nastaju kada atomi liganda doniraju svoj slobodni elektronski par iz s ili p orbitala u slobodnu odgovarajuću hibridizovanu orbitalu akceptorskog atoma. Ukoliko je u pitanju višeatomski ligand, odnosno molekul likganda, ligand može imati jedan ili više donorskih atoma i prema broju donorskih atoma razlikuju se monodentatni (1), bidentatni (2), tridentatni (3), tetradentatni (4) i polidentatni ligandi. Što više donorskih atoma ima jedan ligand to je veza između njega i koordinativnog centra jača. Ligandi se mogu izmenjivati ukoliko se pojave ligandi koji imaju veći afinitet ka vezivanju sa centralnim atomom i oni ne mogu disosovati iz koordinativne sfere bez istiskivanja od strane jačeg liganda. Ovo svojstvo kompleksa metala već je iskorišćeno u "drug delivery" sistemu za azot(II)-oksid putem kompleksa nikla i bakra [47, 48]. Takođe, zbog ovoga kompleksi metala koji su stabilni često imaju drugačija svojstva od prostog zbira svojstava metala i liganada iz kojih su nastali. Ilustrativan primer je tzv. "berlinsko plavo", $Fe_4[Fe(CN)_6]_3$, koje sadrži 6 cijanidnih jona, ali ne pokazuje toksičan efekat koji je karakterističan za cijanidni jon. Biomolekuli koji sadrže atome azota, kiseonika i sumpora predstavljaju dobre kandidate za ligande metala koji grade komplekse. Prilikom razmatranja afiniteta liganda za vezivanje za atom ili jon metala važno je imati u vidu spektrohemijski niz liganada – niz u kojem su ligandi poređani prema jačini odnosno sposobnosti da dovedu do razlaganja ligandnog polja tj. razlaganja odgovarajućih hibridizovanih orbitala centralnog atoma [49]. Spektrohemijski niz sa nekim važnim ligandima dat je kao:

$I^- < Br^- < S^{2-} < SCN^- < C^- < NO_3^- < N_3^- < F^- < OH^- < H_2O < NCS^- < CH_3CN <$ py (piridin) $< NH_3 <$en (etilendiamin) $< NO_2^- < PPh_3 < CN^-$

Ono što je dodatno značajno sa strukturnog aspekta je jaka težnja kompleksa metala da zauzimaju pomenute geometrijske strukture i time mogu modifikovati strukturu vezanog biomolekula. Sa apekta biologije, promene u strukturi impliciraju promene u funkciji, te formiranje bioogranskih koordinativnih jedinjenja može imati značajne posledice po žive sisteme. Zajedno sa svojstvom kompleksa da izmene način na koji se ligandi kasnije ponašaju u reakcionom medijumu ili biološkoj sredini, ovo svojstvo predstavlja okosnicu ideje da se kompleksna jedinjenja nađu među atraktivnim molekulskim modulatorima funkcije biohemijskih sistema, što je prvi korak ka razvoju potencijalnih terapeutskih sredstava [50].

2.7. Kompleksi plemenitih metala u medicini

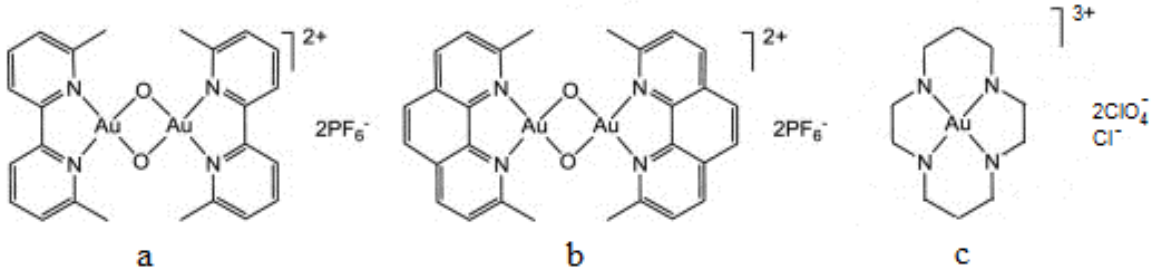
Istorijat upotrebe kompleksnih jedinjenja u medicinske svrhe je dug, potiče još iz viktorijanske ere kada su kao antidoti za trovanja teškim metalima korišćeni kompleksi poput pomenutog "berlinskog plavog" koji imaju sposobnost da koordinativno vežu i na taj način neutrališu toksične vrste. Veliki značaj dobijaju u prvoj polovini dvadesetog veka kada je otkrivena njihova sposobnost da ubijaju bakterije, a nedugo zatim opet kada je otkrivena njihova sposobnost da ubijaju i eukariotske ćelije [51, 52]. Najveću pažnju privukao je kompleks platine *cis*-diammindihlorplatina(II) poznatiji po trgovačkom nazivu cisplatin. Naime, 1978. godine on je odobren kao lek u hemoterapiji kancera jajnika i testisa i pokazao se kao veoma uspešan. Modernizacijom primene uspešnost lečenja raka testisa porasla je i do 85% [53, 54]. Ipak, brojni sporedni efekti koji su nastajali kontinuiranom primenom jasno su pokazali da cisplatin nije konačni ishod istraživanja u toj oblasti već samo važan prvi korak. Dalja istraživanja pokazala su da d^8 kompleksi koji imaju kvadratno planarnu strukturu ili oktaedarsku strukturu sa dobrim odlazećim grupama daju najbolje citotoksične efekte. Mehanizam delovanja je najbolje proučen na cisplatinu, a smatra se da slični kompleksi imaju istovetan mehanizam delovanja. Neposredno po ulasku u organizam, cisplatin izmenjuje jedan od svojih hloridnih jona za molekul vode. Nastali

$[PtCl(H_2O)(NH_3)_2]^+$ kompleks ulazi u ćelije i dolazi do jedra, gde se vezuje za DNK i to dominantno preko gvanidina. Nakon tog vezivanja izmenjuje se i drugi hloridni atom i dolazi do formiranja tzv. "čvora" u lancu DNK koji predstavlja fizičku barijeru replikaciji i rezultuje ćelijskom smrću. Iako je u početku pokazao velike uspehe, negativni aspekti upotrebe ovog leka brzo su postali evidentni. Pojedini tipovi kancera mogu zasad nerazjašnjenim mehanizmima razviti rezistenciju na ovaj tip hemoterapije [55-57]. Srodni kompleksi platine poput oksaliplatina mogu zaobići rezistenciju, ali ih prate isti sporedni efekti, a to su neuro-, nefro-, mijelo-, oto- i hemotoksičnost, zatim izazivanje mučnine i povraćanja, anemije, gubitka elektrolita i brojni drugi. Ovi efekti proizilaze upravo iz male selektivnosti kompleksa za ćelije raka i njihovu DNK. Pokazano je da se kompleksi vezuju i za zdrave ćelije i za njihove proteinske elemente i da remete i njihov metabolizam i dovode do sekundarnih patoloških stanja i neželjenih efekata [58]. Sve ovo dovelo je do istraživanja drugih sličnih metala kao kandidata za razvoj antitumorskih lekova.

Zlato i jedinjenja zlata su dugo bila atraktivna u tom pogledu, ali rezultati u *in vitro* studijama na DNK su izostajali ili nisu bili dovoljno obećavajući da bi se razvili eventualni kandidati za ulazak u kliničke studije. Kompleksi zlata jednostavno nisu pokazivali dovoljno jaku sklonost ka vezivanju sa DNK. S druge strane, kompleksi zlata pokazuju izuzetan afinitet za vezivanje sa biomolekulima koji sadrže sumpor, poput proteina. Ovo svojstvo omogućava da neka od ovih jedinjenja budu enzimski inhibitori [59].

Početno jedinjenje u hemiji kompleksnih jedinjenja zlata je najstariji poznati kompleks zlata – tetrahloroauratni(III) jon $[AuCl_4]^-$. Tetrahloroauratna(III) kiselina $H[AuCl_4]$ nastaje oksidacijom elementarnog zlata u tzv. "carskoj vodi", smeši koncentrovane azotne i klorovodonične kiseline u zapreminskom odnosu 1:3. Tetrahloroauratna(III) kiselina je žuta kristalna supstanca za koju je karakteristično da je izuzetno dobro rastvorljiva u svakom nereagujućem rastvaraču koji sadrži kiseonik, čak i u višim alkoholima koji se slabo mešaju sa vodom ili nepolarnim organskim rastvaračima poput etra. Gradi nerastvorne ili teško rastvorne soli sa većinom metala. Glavna primena tetrahloroauratne(III) kiseline je kao oblik za ekstrakciju zlata iz rude koji se potom podvrgava elektrolizi.

Tetrahloroauratni(III) jon je kvadratno planarne strukture, analogno strukturi cisplatina. Karakteristika ovog jona jeste da njegovi hloridni ligandi lako odlaze i da on vrlo lako podleže hidrolizi i redukciji, naročito u prisustvu organskih liganada koji sadrže sumpor, poput tiouree, cisteina, metionina i glutationa. Sam tetrahloroauratni(III) jon je pokazao visoku toksičnost na biološkim organizmima [60, 61]. Smatra se da se mehanizmi toksičnog delovanja ne razlikuju od mehanizama opšteg toksičnog delovanja jona teških metala, jer je ovaj kompleks u životinjskom organizmu nestabilan i brzo se raspada na jone zlata i odgovarajuće kontra jone [62]. Razlog zbog kojeg je tetrahloroaurat(III) ipak od značaja za medicinsku upotrebu kompleksnih jedinjenja leži u činjenici da je on polazno jedinjenje za sintezu cele familije novih kompleksnih jedinjenja koja danas postoje [63]. Zbog male stabilnosti u biološkoj sredini, kompleksi zlata su dugo zanemarivani, međutim nedavna istraživanja utvrdila su postojanje malog broja mononuklearnih kompleksa zlata koji su dovoljno stabilni u biološkoj sredini da bi mogli da posluže kao polazna tačka za razvoj novih antitumorskih terapija. Ovde se pre svega misli na komplekse sa dimetil sulfoksidom (DMSO), poput $[AuCl_2(DMSO)_2]^+$. Naročito su obećavajuća jedinjenja sa velikim organskim bidentatnim, tridentatnim ili čak tetradentatnim ligandima koja su pokazala daleko manju toksičnost i veću specifičnost u biološkom delovanju, kao i izuzetnu stabilnost u biološkim uslovima. Ova jedinjenja su razvijana sa ciljem da se toksično delovanje zlata usmeri ka tumorskim ćelijama i procesima koji su od vitalnog značaja za njihov rast i deobu. Bipiridinski kompleksi, kako mononuklearni, tako i dinuklearni su obećavajuća grupa kompleksa zlata. Upotrebom 2,2'-bipiridina i njegovih derivata lako se dobijaju *cis* kompleksi koji su analogni cisplatinu ili pak dinuklearni kompleksi poput novijih Au-oxo6 i Au_2Phen kompleksa prikazanih na slici 5. Krunični ligandi i kriptandi takođe predstavljaju jedan od pravaca u istraživanju kompleksa zlata sa namerom da se "maskira" prava reaktivna priroda jona zlata [64-66].



Slika 5. Primeri dinuklearnih i kriptandskih kompleksa zlata. a - Au-oxo6, b - Au_2Phen, c - Aucyclam.

Neki od ovih kompleksa su već pokazali biološka dejstva. Aucyclam je pokazao afinitet za vezivanje sa DNK i uticao je na promenu konformacije DNK. Au-oxo6 i Au2Phen koji su dinuklearni kompleksi pokazali su citotoksično delovanje na kulturi HeLa-60 ćelija. AubipyC je pokazao sklonost ka vezivanju za važne regulatorne proteine u ćeliji kao što je citohrom c. Postoje i brojni drugi primeri biološke aktivnosti ovih i sličnih kompleksa, ali još uvek ne postoje proizvodi u redovnoj upotrebi [67, 68].

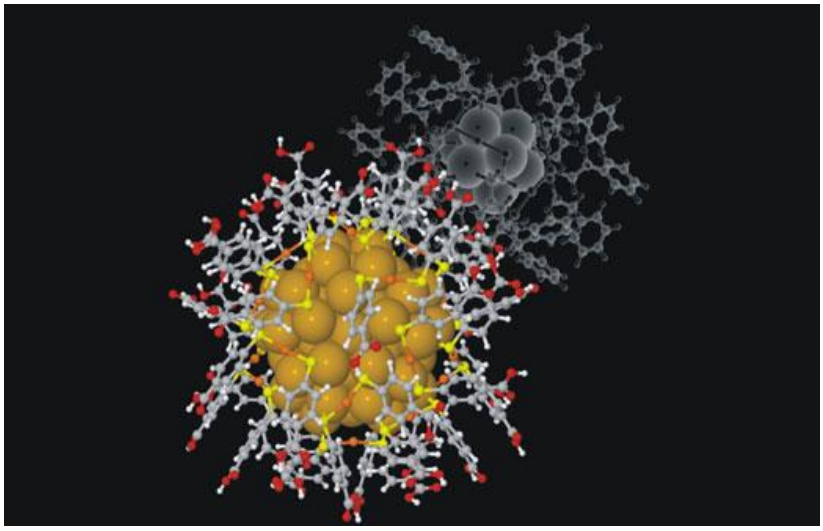
Kao što je nekoliko navedenih studija pokazalo, kompleksi zlata mogu da interaguju sa DNK, ali su skloniji reakciji sa proteinima. Smatra se da svoje citotoksično dejstvo ispoljavaju po vezivanju za protein menjajući mu strukturu a time i funkciju, ali se malo zna o događajima do kojih dolazi nakon vezivanja kompleksa zlata za protein. Nekoliko studija je pokazalo da se vezivanje odigrava preko –SH grupa na cisteinu, a poznato je da interakcija sa –SH grupama može izazvati redukciju jona zlata Au(III) do zlata Au(I) ili čak elementarnog zlata, što za uzvrat može imati kao posledicu formiranje nanočestica zlata, koje su takođe biološki aktivne [11, 69, 70].

2.8. Struktura i primena nanočestica zlata

Nanočestice zlata predstavljaju koloidne agregate atomskog zlata, jona zlata i odgovarajućih kontra jona. Postoji više vrsta koloidnih nanočestica zlata koje se međusobno razlikuju prema obliku, veličini, sastavu i svojstvima. Prema obliku nanočestica

zlata razlikuju se nanosfere, nanožice, nanoštapići, nanocevi, itd. Postoji više načina sinteze nanočestica i način sinteze u mnogome određuje tip kontra jona. Iako je istorijski najstariji metod redukcije zlata i formiranja koloida zlata sa polovine devetnaestog veka podrazumevao upotrebu fosfora, najčešće korišćen metod koristi citratne jone kao redukciono sredstvo. U takvim nanočesticama javlja se citrat kao kontra jon, jer se citrat dodaje u višku. Postoje i brojne druge metode koje koriste različita redukciona sredstva i tehnike da bi se formirale nanočestice i od metode koja se koristi i od uslova u reakciji zavise i karakteristike gotovih nanočestica. Mogu se koristiti natrijum borhidrid, organski rastvarači poput toluena, hidrohinon, tioli, šećeri i mnogi drugi redukcioni agensi. Postoji puno opisanih metoda za sintezu široke palete različitih tipova nanočestica zlata [11, 71-78].

Struktura većine običnih, nemodifikovanih, sfernih nanočestica zlata je klasterska, tj. u sastav jedne nanočestice ulaze i joni i atomi zlata kao i odgovarajući kontra jon. Centralni deo nanočestice čini pretežno atomsko i jonsko zlato dok se bliže površini na granici koloidne sfere javlja sloj kontra jona (slika 6.). Na površini nanočestice prisutne su sve tri vrste i to je od značaja za biološko delovanje nanočestica [79].



Slika 6. – Struktura sferne nanočestice zlata

Rastvori koliodnog zlata su najčešće rastvori crvenkaste boje. Ovo je posledica fenomena površinske plazmonske rezonancije. Površinska plazmonska rezonancija je kvantni fenomen do kojeg dolazi kada foton dolazećeg svetlosnog zraka pogodi površinski sloj atoma nekog metala, u ovom slučaju zlata, i biva moduliran frekvencijom njegovog poslednjeg elektronskog sloja, te mu se tako menja i frekvencija, tj. u slučaju vidljive svetlosti menja mu se boja. Ovaj fenomen je danas u upotrebi u osetljivoj analitici protein-protein i protein-ligand interakcija. Osim toga, površinska plazmonska rezonancija daje koloidnim rastvorima važnu makroskopsku karakteristiku koja se može koristiti za proučavanje interakcije čestice i nekog drugog molekula [80].

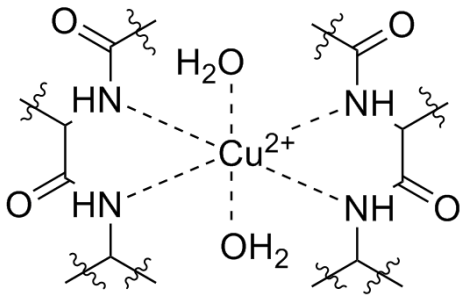
Veličina nanočestica koje se dobijaju navedenim metodama sinteze kreće se od približno 5 do 50 nm, premda postoje i veće nanočestice. Biološka aktivnost nanočestica je dobro ispitana i poznato je da deluju citotoksično, baktericidno, viricidno, kao i da mogu biti modifikovane vezivanjem selektivnih bioloških markera u svrhe osetljive dijagnostike tumorskih i autoimunskih oboljenja. Manje nanočestice su pokazale veću biološku aktivnost, ali je idalje nejasno da li je uzrok tome veličina ili sastav ovih čestica. Osim toga, nije dublje istraživano da li su za interakciju sa biološkim materijalom odgovorne jonske ili atomske vrste zlata [70, 81-86].

2.9. Interakcije metala i proteina

Proteini sadrže veliki broj reaktivnih funkcionalnih grupa koje i tokom normalnog metabolizma koriste za inerakcije sa metalima. Naročito specijalizovani proteini kao što su jonske pumpe i signalni receptori imaju specijalno rezervisana mesta za vezivanje odgovarajućih metala ili njihovih jona. Jedan od primera je već pomenuta Na^+/K^+ ATPaza koja selektivno vezuje jone natrijuma i kalijuma u regionu koji je bogat hidrofobnim i negativno naelektrisanim ostacima aminokiselina. Iako sam mehanizam vezivanja jona nije u potpunosti poznat, smatra se da je reč o takozvanim sonim mostovima koji predstavljaju lokalno formirano jonsko jedinjenje u nepolarnoj sredini. Ovo je čest slučaj interakcije

između natrijuma i proteina jer natrijumov jon ima sposobnost da veže veliku količinu vode preko indukovanog dipola i stoga proteini koji interaguju sa natrijumom tu interakciju uspostavljaju upravo u hidrofobnoj sredini [87]. Poznato je da su za vezivanje natrijuma i kalijuma od ključnog značaja ostaci serina i asparaginske kiseline i to Ser755, Asp804 i Asp808. Ovo svakako nisu jedine aminokiseline uključene u proces jer je poznato da mutacije u transmembranskim regionima takođe remete funkciju enzima [19, 88].

Zemnoalkalni joni, poput kalcijumovog jona, veoma su skloni vezivanju za karboksilne grupe bočnih ostataka poput onih na asparaginskoj i glutaminskoj kiselini. Kalmodulin je najpoznatiji metaloprotein koji vezuje kalcijum i to radi upravo preko kiselih bočnih ostataka [89]. Teži metali, poput gvožđa i bakra su jako reaktivni i vezivanje proteina sa njima obično se odvija preko prostetičnih grupa kao posrednika. Ilustrativan primer je hemoglobin, gde je gvožđe koordinativno vezano u porfirinski prsten koji je dalje vezan za protein. Ipak, čak i tu jedan od liganada gvožđa je azot iz proksimalnog histidinskog ostatka, što pokazuje da je i azotov atom dobar kandidat za interakciju sa metalima [90]. Izvan metabolizma, u proteinskoj hemiji postoje bojene reakcije poput biuretske reakcije i Lorijeve (Lowry) metode za bojenje proteina bakrom [91]. U oba slučaja nastaje kompleks bakra, dva molekula vode i četiri amidna azota iz peptidne veze i taj kompleks je ljubičaste boje koja je merljiva na spektrofotometru na 540 nm. U uslovima biološke sredine, prisustvo bakra može dovesti do vezivanja za proteine i druge biomolekule i do oksidativnog oštećenja na njima.



Slika 7. Kompleks bakra i peptidnog lanca koji nastaje u biuretskoj reakciji.

U slučaju toksičnih teških metala poput žive, olova, kadmijuma, talijuma i drugih glavnu ulogu u vezivanju sa proteinima igraju aminokiseline koje sadrže sumpor, cistein i metionin [92-95]. Druge aminokiseline takođe mogu da učestvuju u vezivanju ovih metala. Poznato je da ovi metali grade jake i nerastvorne komplekse sa jedinjenjima sumpora i njihovo vezivanje i prateća redukcija do atomskih oblika praćeni su gubitkom strukture i funkcije proteina ili drugih biomolekula. Njihova sposobnost da izazovu oksidativna oštećenja u biomolekulima počiva upravo na pomenutoj redukciji jona metala [96, 97].

Kada je reč o plemenitim metalima, njihova mala aktivnost u atomskom obliku čini ih relativno inertnim u pogledu vezivanja za biomolekule, premda atomski oblici zlata i srebra mogu graditi stabilne komplekse sa pomenutim aminokiselinama koje sadrže sumporov atom. Iz laboratorijske prakse dobro je poznato da slučajno izlaganje kože rastvoru srebro nitrata dovodi do pojave tamno smeđih i crnih fleka po koži. Upravo ova reakcija posledica je vezivanja jona srebra za proteine preko atoma sumpora i nastanka srebro sulfida i redukcije do atomskog srebra. Joni plemenitih metala su veoma reaktivni, takođe se preferencijalno vezuju za proteine preko sumpora, ali je poznato da mogu da se vezuju i preko azotovog atoma. Ovo je naročito važno za razvoj terapeutika koji se vezuju za DNK, kao pomenuti cisplatin, gde se vezivanje ostvaruje upravo preko azota u bazama DNK [55].

Pojedini joni metala su neophodni za funkciju proteina, naročito u slučaju kofaktora enzima, poput kalcijuma u amilazi. Pojedini tipovi amilaze bez kalcijuma ne mogu da obavljaju svoju funkciju [98]. Enzimi uključeni u sintezu nukleinskih kiselina ne mogu da obavljaju svoju funkciju bez prisustva magnezijuma. U pojedinim slučajevima interakcja između proteina i jona metala odigrava se preko ostataka šećera na proteinu ili preko fosfatne grupe vezane na proteinu [95]. U slučaju regulatornih proteina DNK postoje tzv. "cinkovi prsti" u kojima glavnu strukturnu komponentu čini region proteina koji je vezao jon cinka i time formirao jedinstveni strukturni motiv koji mu omogućava regulatornu funkciju. Postoje eksperimentalni podaci koji govore o tome da bi joni kobalta mogli da zamene cink u obavljanju ove funkcije [99, 100]. Sve ovo govori o tome da je interakcija između metala i proteina ne samo značajna za normalan ćelijski metabolizam, već da može u potpunosti diktirati preživljavanje ili odumiranje ćelije. Biološke implikacije hemijske

reakcije između proteina i drugih biomolekula sa jonima ili atomima metala izuzetno su velike i mogu činiti razliku između preživljavanja ili smrti jedinke u celosti. Čovekova sposobnost da preko ove interakcije selektivno utiče na metaboličke procese značila bi mogućnost za formulisanje novih terapija oboljenja koja su posledice abnormalnog metabolizma ćelije.

3. Cilj rada

Cilj ove studije bio je da se ispita priroda eventualne interakcije prikazanih kompleksa i nanočestica zlata sa preparatima Na^+/K^+ ATPaze. Pre svega, zadatak je bio utvrditi da li postoji inhibicija enzima ovim jedinjenjima zlata, jer su ranija istraživanja pokazala da postoji sklonost ovih jedinjenja ka vezivanju sa proteinima, a naročito ukoliko ti proteini poseduju –SH grupe, a poznato je da Na^+/K^+ ATPaza obiluje slobodnim –SH grupama [19, 37, 88].

Sledeći zadatak bio je ispitati prirodu te inhibicije. Ranije studije pokazale su da kompleksi metala koji deluju na Na^+/K^+ ATPazu dovode do nekompetitivne inhibicije, te je ovde očekivan isti ili sličan rezultat. Pored toga jedna od ideja je bila i da se proveri može li se eventualna inhibicija koju jedinjenja zlata izazivaju sprečiti ili otkloniti dodavanjem jedinjenja koja bi bila kompetirajući donori –SH grupa, naime cisteinom i glutationom. Ukoliko je to moguće, cilj je bio i da se ispita priroda te reaktivacije inhibiranog enzima kao i sprečavanja inhibicije.

Osim ovoga cilj je bio i da se u uslovima simulirane biološke sredine, kao što je izolovana humana krv, ispita da li ovi kompleksi zadržavaju sposobnost da inhibiraju enzim. U realnim biološkim uslovima postoji mnogo drugih molekulskih vrsta koje bi mogle da kompetiraju za vezivanje sa kompleksom, pa je važno proveriti njihovu selektivnost za Na^+/K^+ ATPazu i stabilnost u tim uslovima.

Najzad, cilj je bio i da se utvrdi da li se interakcija između nanočestica i Na^+/K^+ ATPaze može eksploatisati na druge načine i upotrebiti za povećanje osetljivosti određenih tipova enzimskih testova.

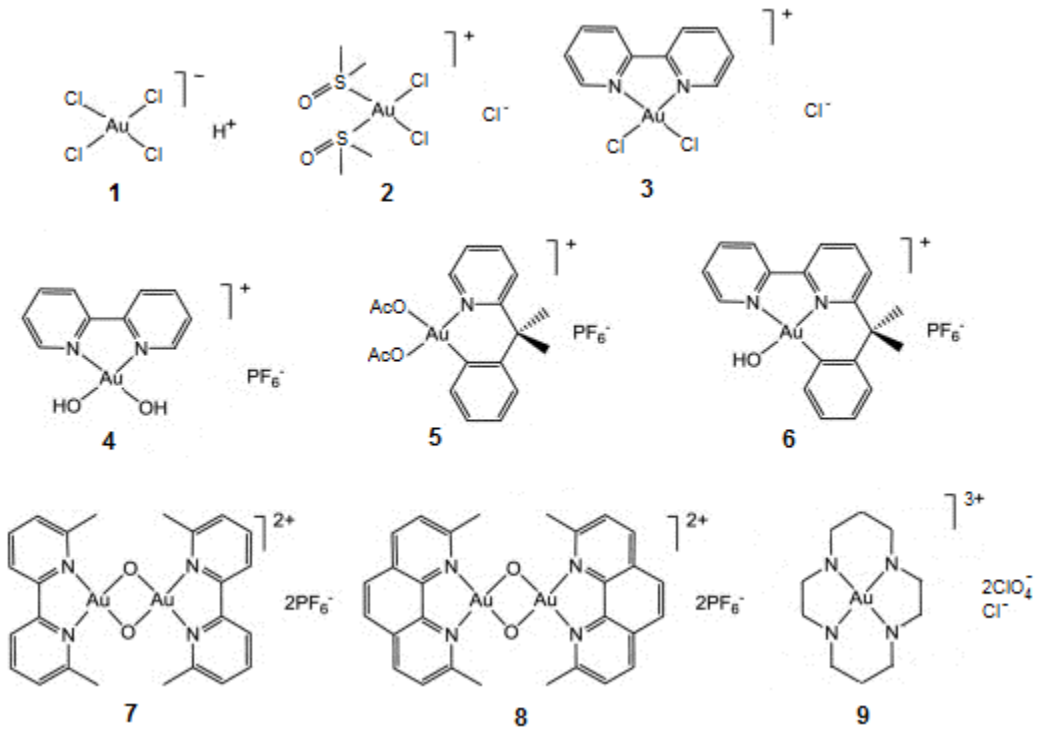
NAŠ RAD

4. Rezultati

4.1. Ispitivana jedinjenja zlata

Ovom studijom obuhvaćeno je devet kompleksa zlata i tri tipa koloidnih nanočestica zlata, i to:

Kompleksi: $H[AuCl_4]$ (skraćeno $AuCl_4$, Aldrich), $[Au(DMSO)_2Cl_2]Cl$ (skraćeno AuDMSO, sintetisan prema referenci [101]), $[Au(bipy)Cl_2]Cl$ (skraćeno Aubipy, sintetisan prema referenci [64]), $[Au(bipy)(OH)_2][PF_6]$ (skraćeno AubipyOH, sintetisan prema referenci [67]), $[Au(py^{dmb}\text{-}H)AcO)_2][PF_6]$ (skraćeno Aupy, gde je py^{dmb}-H = deprotonovani 6-(1,1-dimetilbenzil)-piridin, sintetisan prema referenci [102]), $[(bipy^{dmb}\text{-}H)Au(OH)][PF_6]$ (skraćeno AubipyC, gde je $bipy^{dmb}$-H = deprotonovani 6-(1,1-dimetilbenzil)-2,2'-bipiridin, sintetisan prema referenci [67]), $[(bipy^{2Me})_2Au_2(\mu\text{-}O)_2]$ (skraćeno Au-oxo6, gde je $bipy^{2Me}$ = 6,6'-dimetil-2,2'-bipiridin, sintetisam prema referenci[103]), $[(phen^{2Me})_2Au_2(\mu\text{-}O)_2][PF_6]_2$ (skraćeno Au_2phen, gde je $phen^{2Me}$ = 2,9-dimetil-1,10-fenantrolin, sintetisan prema referenci [103]) i $[Au(cyclam)](ClO_4)Cl_2$ (skraćeno Aucyclam, gde je cyclam = 1,4,8,11-tetraazaciklotetradekan, sintetisan prema referenci [65]). Svi kompleksi su čuvani u suvom stanju do rada, a koncentrovani radni rastvori pravljeni su u koncentraciji od 10^{-2} M u DMSO ili acetonitrilu, u zavisnosti od rastvorljivosti kompleksa. Struktune formule kompleksa su date na slici 8.

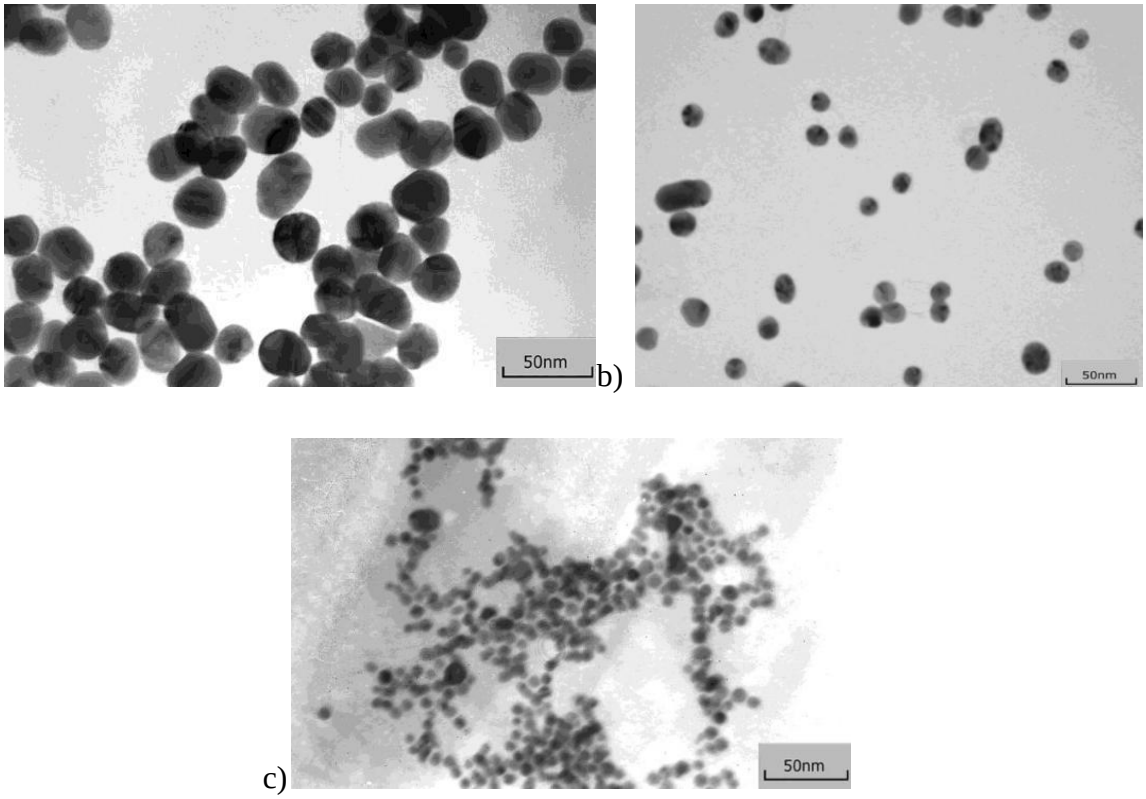


Slika 8. – Strukturne formule ispitivanih kompleksa. 1 – $[AuCl_4]^-$; 2 – AuDMSO; 3 – Aubipy; 4 –AubipyOH; 5 – Aupy; 6 – AubipyC; 7 – Au-oxo6; 8 – Au_2phen; 9 – Aucyclam.

Koloidi zlata su sintetizovani prema sledećim metodama i čuvani u frižideru do rada: Koloid 1 (C1, dijametar nanočestice 30 ± 0,8 nm) i koloid 2 (C2, dijametar nanočestice 15 ± 0,7 nm) su sintetisani prema modifikovanoj ranije opisanoj metodi [104]. Naime, 200 mL 1 mM $HAuCl_4$ je zagrejano do ključanja uz energično mešanje u balonu sa okruglim dnom na koji je postavljen refluks kondenzator. Potom je u rastvor brzo dodato 20 mL 38,8 mM rastvora trinatrijum-citrata. Rastvor je ključao još 15 minuta, a zatim je ohlađen do sobne terperature uz konstantno mešanje.

Koloid 3 (C3, dijametar nanočestice 9,5 ± 0,8 nm) sintetisan je prema ranije opisanoj metodi [105]. Ove nanočestice su sintetisane redukcijom 100 mL 0,2 mM vodenog rastvora $KAuCl_4$ sa $NaBH_4$, čija je finalna koncentracija u rastvoru bila 5,3 mM, na sobnoj temperaturi.

Koncentracije radnih rastvora koloida određene su spektrometrijski uz upotrebu literaturnih podataka za molske apsorpcione koeficijente [106, 107]. Dijametri nanočestica potvrđeni su na transmisionom elektronskom mikroskopu (slika 9.). Finalne koncentracije rastvora su bile $1{,}8 \times 10^{-7}$ M za C1, $1{,}11 \times 10^{-8}$ M za C2 i $2{,}8 \times 10^{-7}$ M za C3. Sve koncentracije su podešene na $1{,}8 \times 10^{-7}$ M radi uporedivosti dobijenih rezultata.

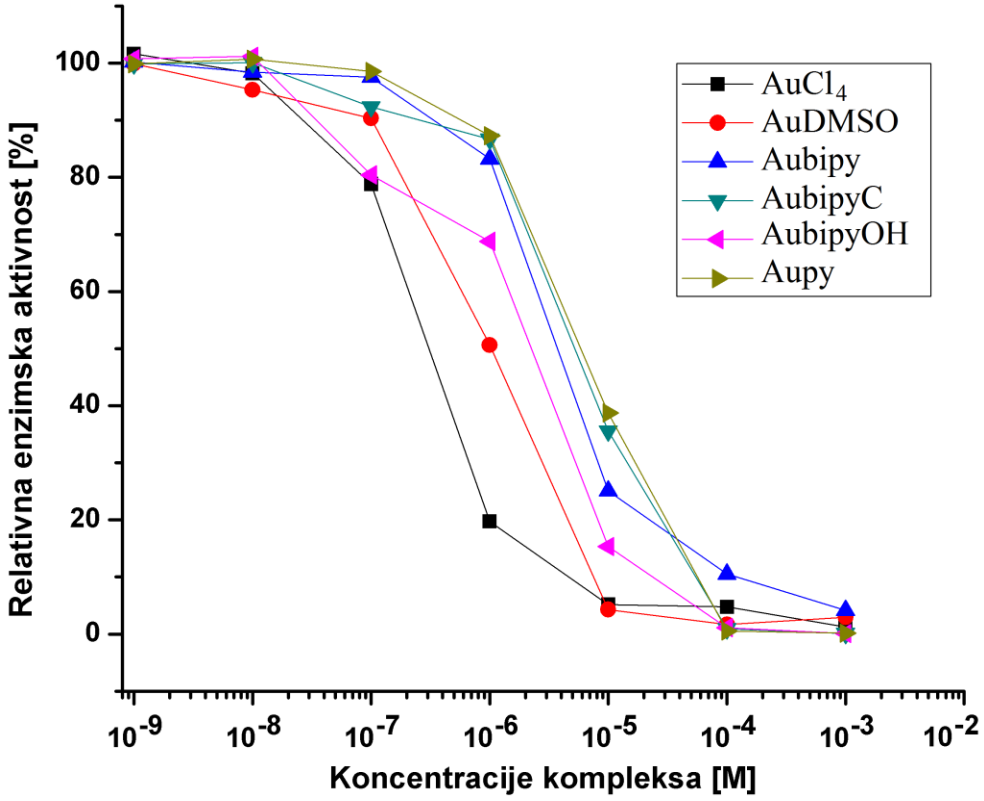


Slika 9. – Mikrografije sa transmisione elektronske mikroskopije. a) Koloid C1, b) koloid C2, c) koloid C3.

4.2. Kompleksi zlata

4.2.1. Inhibicija enzima kompleksima

Ispitivan je potencijal kompleksa da inhibiraju Na^+/K^+ ATPazu. U test za ispitivanje relativne aktivnosti Na^+/K^+ ATPaze dodavani su rastvori kompleksa u vodi do finalnih koncentracija u opsegu 10^{-8} do 10^{-3} M. Na taj način dobijene su inhibicione krive za sve komplekse i njihove IC_{50} – koncentracije koje dovode do pada relativne aktivnosti enzima za 50%. Inhibicione krive su date na slici 10.



Slika 10. – Inhibicione krive ispitivanih kompleksa zlata.

Kao što se može videti na slici 10, najjači inhibitor je $[AuCl_4]^-$ jon, a ostali kompleksi izuzev kompleksa Au-oxo6, Au_2phen i Aucyclam daju veoma jake inhibicije u opsegu oko 10^{-5} do 10^{-7} M. Istraživanje je nastavljeno sa kompleksima koji su pokazali inhibitorni efekat, pa kompleksi Au-oxo6, Au_2phen i Aucyclam nisu dalje proučavani.

Kao značajan sporedan efekat uočen je pad relativne aktivnosti pratećih i sporednih ATPaza kada su ovi kompleksi ispitivani na modelu SMP i humanih ćelijskih membrana. Pri višim koncentracijama inhibitora (10^{-4} do 10^{-3} M) uočen je pad aktivnosti pratećih ATPaza i do 50%.

4.2.2. Analiza inhibicionih krivih Hilovom metodom

Hilovom analizom dobijenih krivih izračunati su Hilovi koeficijenti, prema sledećoj formuli:

$$\log\left(\frac{REA}{100-REA}\right) = -n \times \log[I] + n \times logIC_{50}$$

gde je n Hilov koeficijent, REA je procenat relativne enzimske aktivnosti, a I je koncentracija inhibitora u mol/L, a IC_{50} je koncentracija inhibitora koja daje 50% inhibicije. Vrednosti za IC_{50} za ispitivane komplekse očitane su sa grafika inhibicije enzimske aktivnosti nakon sigmoidnog fitovanja krivih i date su u tabeli 1. zajedno sa izračunatim vrednostima za Hilove koeficijente.

Tabela 1. - Vrednosti i Hilovog koeficijenta (n) za ispitivane komplekse.

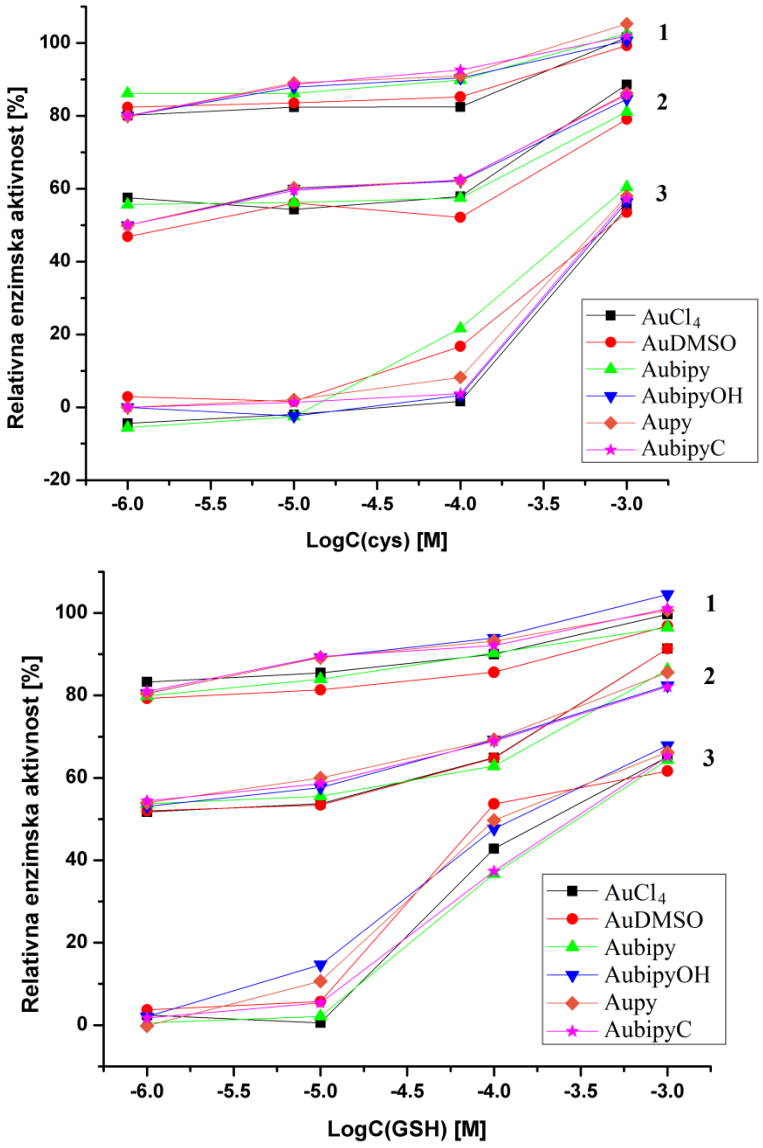
Kompleks	IC_{50} [M]	n
$AuCl_4$	$(7{,}19 \pm 0{,}02) \times 10^{-7}$	5,26 ± 0,01
AuDMSO	$(1{,}50 \pm 0{,}02) \times 10^{-6}$	2,25 ± 0,01
Aubipy	$(5{,}88 \pm 0{,}02) \times 10^{-6}$	4,51 ± 0,01
AubipyOH	$(3{,}51 \pm 0{,}02) \times 10^{-6}$	2,81 ± 0,01
Aupy	$(7{,}60 \pm 0{,}02) \times 10^{-6}$	2,37 ± 0,01
AubipyC	$(6{,}63 \pm 0{,}02) \times 10^{-6}$	1,83 ± 0,01

Vrednosti Hilovog koeficijenta ukazuju na prisustvo pozitivne kooperativnosti u vezivanju kompleksa zlata za enzim. Vezivanje jednog molekula kompleksa povećava afinitet enzima ka vezivanju sledećeg, dok se sva vezivna mesta ne popune.

4.2.3. Sprečavanje inhibicije dodatkom donora –SH grupe

Jedna od hipoteza o mehanizmu inhibicije tokom rada bila je da se vezivanje između enzima i kompleksa zlata odvija preko –SH grupa na bočnim aminokiselinskim ostacima cisteina, kojima enzim obiluje. Jedan od načina da se ovo proveri jeste da se u enzimski test pre dodatka enzima, a posle dodatka kompleksa doda neki donor –SH grupe i da se prati da li dolazi do sprečavanja inhibicije na ovaj način. U enzimski test je pre dodatka enzima, a posle dodatka kompleksa pri koncentracijama jednakim IC_{20}, IC_{50} i IC_{100} dodavan i cistein ili redukovani glutation (GSH) u opsegu koncentracija od 10^{-6} do 10^{-3} M, urađena je dodatna preinkubacija od 10 minuta na 37 ºC kako bi se ostvarila reakcija između –SH donora i kompleksa. Nakon toga test se odvijao uobičajeno. U svim ispitivanim

slučajevima došlo je do sprečavanja inhibicije u zavisnosti od količine prisutnog –SH donora. Rezultati su prikazani na slici 11.

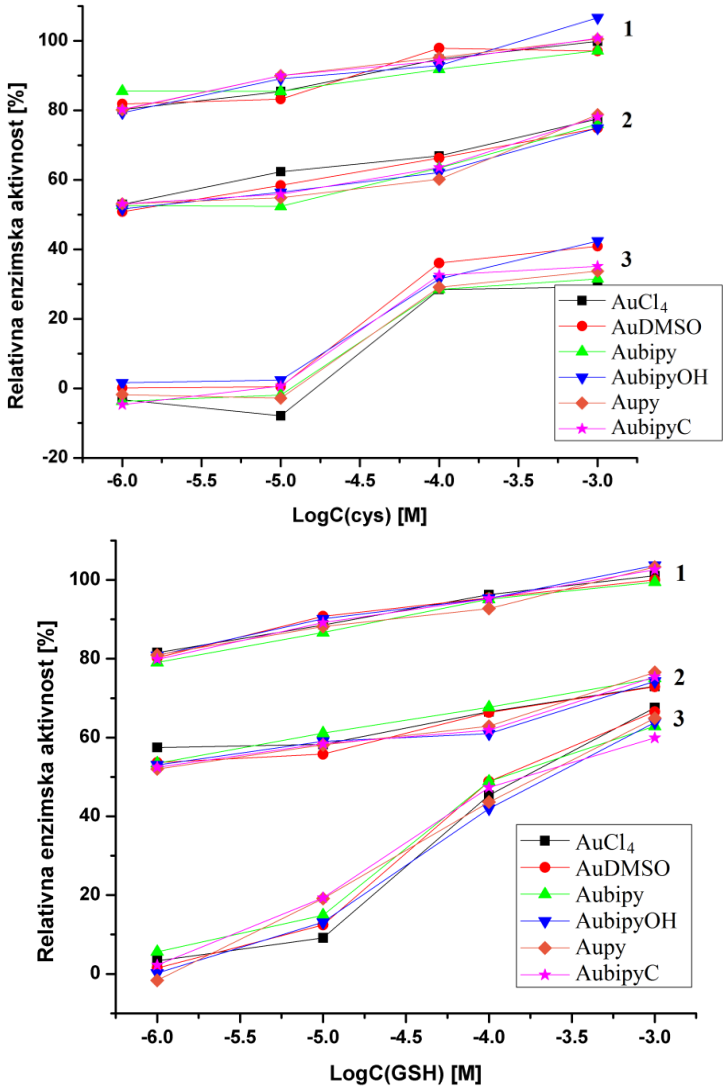


Slika 11. – Grafici sprečavanja inhibicije cisteinom (gore) i glutationom (dole) pri koncentraciji inhibitora od IC_{20} (grupa 1), IC_{50} (grpua 2) i IC_{100} (grupa 3).

Ovo je prilično jak, premda ne i definitivan indikator da se vezivanje kompleksa za enzim odvija preko –SH grupa, najverovatnije izmenom liganda. Ovo će kasnije i definitivno biti pokazano masenim tehnikama.

4.2.4. Reaktivacija inhibiranog enzima

Važno pitanje je bilo da li se inhibicija do koje dolazi po izlaganju enzima kompleksima zlata može otkloniti nakon vezivanja kompleksa za enzim, tj. može li se enzim reaktivirati nakon inhibicije. Stoga je u osnovni test za ispitivanje inhibicije enzima sa koncentracijama kompleksa od IC_{20}, IC_{50} i IC_{100}, ubačen korak u kojem se nakon dodatka inhibitora i enzima u test dodaje i –SH donor, cistein ili glutation, u opsegu koncentracija od 10^{-6} do 10^{-3} M i smeša se inkubira još 10 minuta na 37 ºC, kako bi se inhibitor uklonio sa enzima u prisustvu kompetirajućeg donora –SH grupa. Rezultati ovih testova dati su na slici 12.



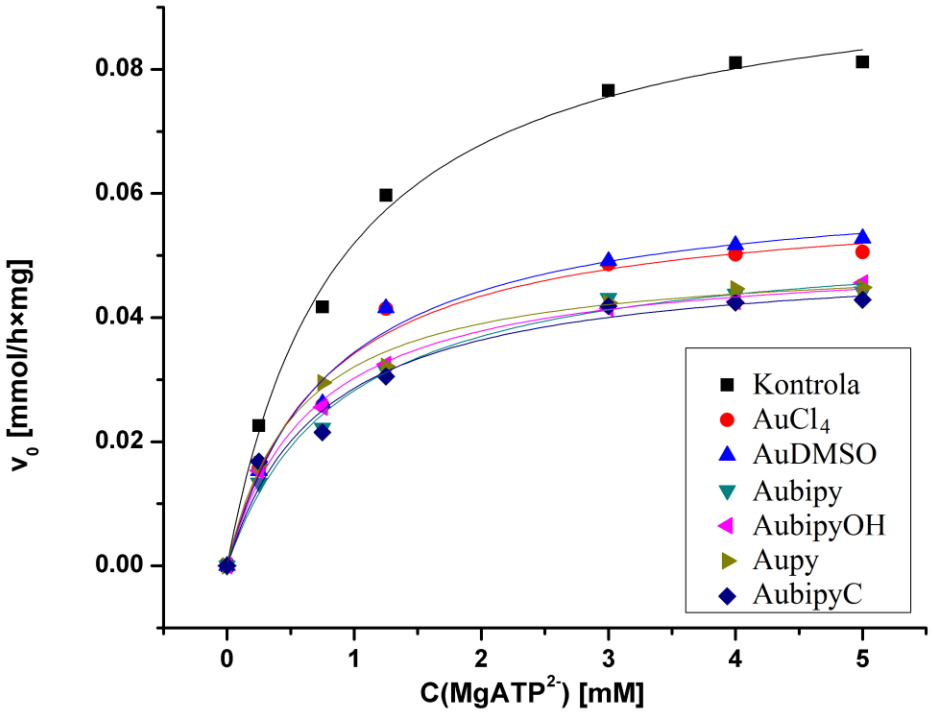
Slika 12. – Grafici reaktivacije inhibiranog enzima cisteinom (gore) i glutationom (dole) pri koncentraciji inhibitora od IC_{20} (grupa 1), IC_{50} (grpua 2) i IC_{100} (grupa 3).

Cistein i glutation mogu reaktivirati enzim koji je inhibiran kompleksima zlata. Ipak, na slikama se uočava da je trend te reaktivacije nešto sporiji nego što je to slučaj kod sprečavanja inhibicije gde je ovaj trend nešto linearniji.

4.2.5. Kinetičke studije

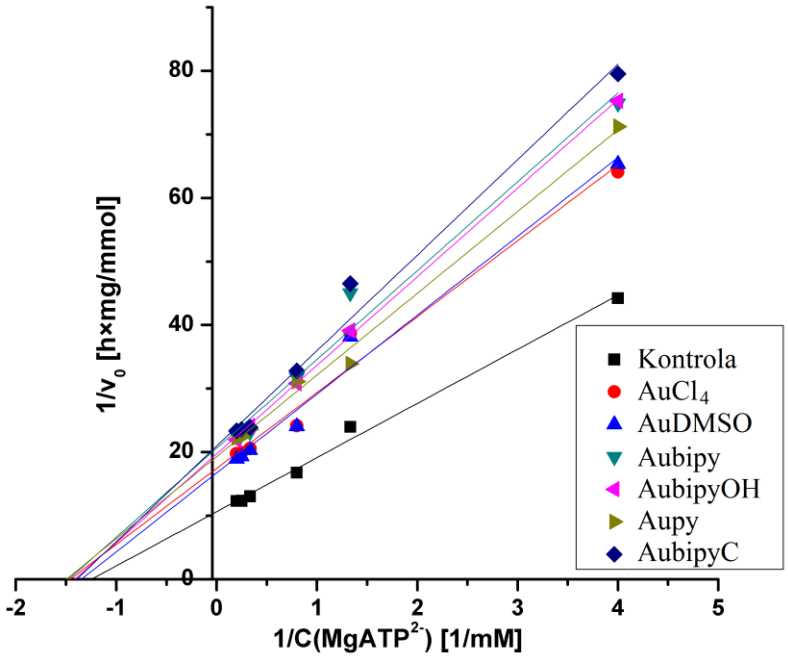
Nakon što je utvrđeno da inhibicija postoji kod ispitivanih kompleksa, pristupilo se određivanju njene kinetike i mehanizma. Upotrebom opisanog enzimskog testa, komercijalnog prečišćenog enzima Na^+/K^+ ATPaze i ispitivanih kompleksa utvrđeni su kinetički parametri i tipovi inhibicije za sve komplekse koji su pokazali inhibitornu aktivnost.

Za svaki od ispitivanih kompleksa urađen je eksperiment pri koncentraciji bliskoj IC_{50}, u kojem je enzim najpre inhibiran pomenutom koncentracijom inhibitora, a zatim mu je dodat supstrat u koncentracijama od 0,25, 0,75, 1,25, 3, 4 i 5 mM. Za svaku od proba reakcija je trajala 4 minuta, nakon čega je zaustavljena i merena prema gore opisanom postupku. Napravljena je kalibraciona kriva za apsolutne vrednosti fosfata, preko koje je izračunata količina nastalog proizvoda. Time su dobijeni podaci za konstrukciju grafika Mihelis-Mentenine krive za svaki ispitivani kompleks i kontrolu. Kumulativni grafik je dat na slici 13.



Slika 13. – Mihelis-Mentenina kriva zavisnosti početne brzine reakcije od koncentracije supstrata.

Kako bi se dobili gore navedeni kinetički parametri K_M i v_{max}, pristupilo se linearizaciji ovog grafika, prema Lajnviver-Barkovoj (Lineweaver–Burk) metodi . Recipročne vrednosti koncentracije supstrata i početne brzine reakcije su iskorišćene za konstrukciju grafika na slici 14.



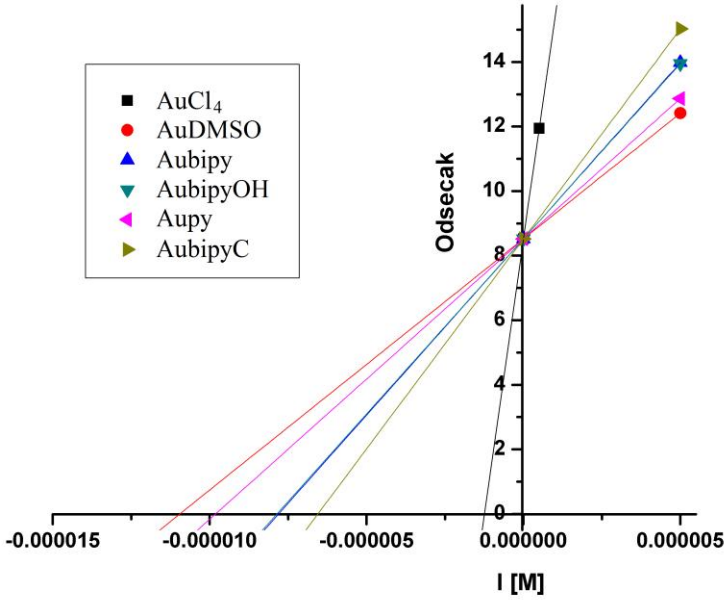
Slika 14. – Lajnviver-Bark linearizacija dobijenih Mihelis-Menteninih krivih.

Računski je određena vrednost za K_M i v_{max} inhibiranog enzima za svaki od ispitivanih kompleksa. Rezultati su prikazani u tabeli 2.

Tabela 2. K_M i v_{max} vrednosti za enzim inhibiran ispitivanim kompleksima.

Kompleks	K_M [mM]	v_{max} [mmol/h/mg]
Kontrola	0,79 ± 0,07	0,094 ± 0,005
$AuCl_4$	0,63 ± 0,10	0,047 ± 0,004
AuDMSO	0,73 ± 0,11	0,059 ± 0,005
Aubipy	0,67 ± 0,11	0,057 ± 0,005
AubipyOH	0,72 ± 0,10	0,052 ± 0,005
Aupy	0,69 ± 0,12	0,050 ± 0,006
AubipyC	0,68 ± 0,09	0,051 ± 0,004

Uočava se da ne dolazi do promene u K_M vrednosti, dok se v_{max} smanjuje. Svi ispitivani kompleksi pokazali su nekompetitivnu reverzibilnu inhibiciju. Kako bi se odredila vrednost konstante inhibicije K_i, konstruisani su sekundarni grafici, dati na slici 15. Konstante inhibicije za sve ispitivane komplekse date su u tabeli 3.



Slika 15. – Sekundarni grafik za određivanje konstanti inhibicije za ispitivane komplekse.

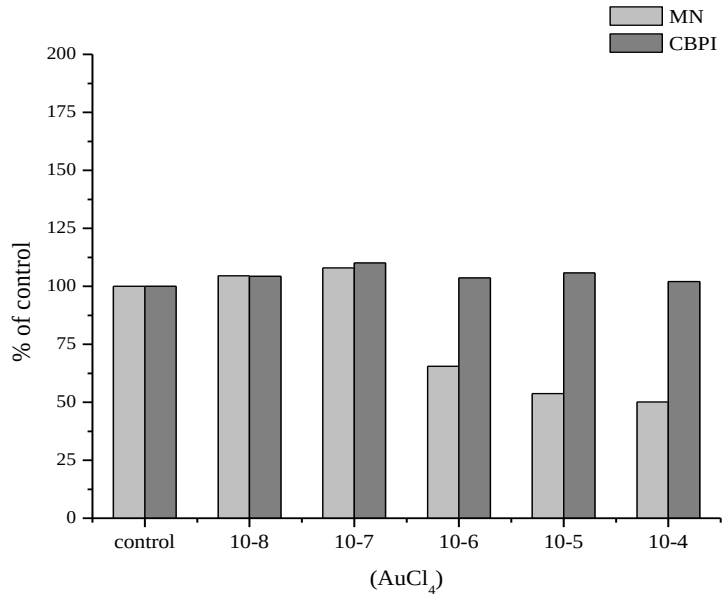
Tabela 3. Konstante inhibicija za sve ispitivane komplekse.

Kompleks	K_i [M]
$AuCl_4$	$1{,}25 \times 10^{-6}$
AuDMSO	$1{,}10 \times 10^{-5}$
Aubipy	$7{,}80 \times 10^{-6}$
AubipyOH	$7{,}86 \times 10^{-6}$
Aupy	$9{,}81 \times 10^{-6}$
AubipyC	$6{,}55 \times 10^{-6}$

Najmanju konstantu inhibicije pokazao je $[AuCl_4]^-$ jon kao što je i očekivano, a AuDMSO pokazao najveću konstantu inhibicije.

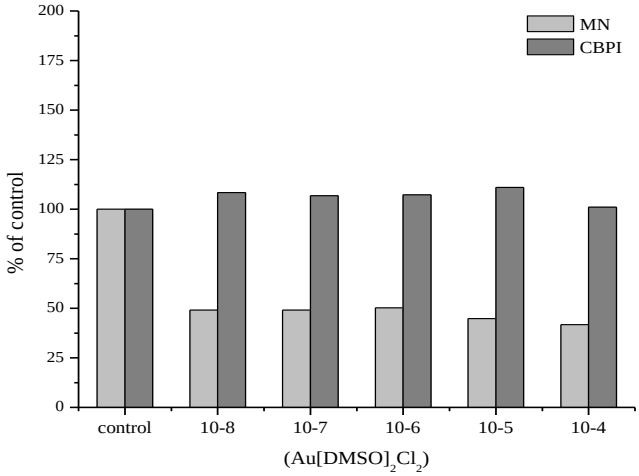
4.2.6. Ispitivanja na ćelijskim kulturama

Radi provere aktivnosti u simuliranim uslovima biološke sredine efekti ispitivanih kompleksa mereni su na kulturama humanih limfocita prema gore opisanoj metodi, a rađena su za komplekse $[AuCl_4]^-$, AuDMSO, Aubipy, AubipyOH, Aupy i AubipyC. Za komplekse Au-oxo6, Au_2phen i Aucyclam efekti na humanim ćelijama su poznati iz ranije publikovanih literaturnih podataka [103]. Praćeni su parametri citotoksičnosti (CBPI i MN), kao i rezidualna aktivnost Na^+/K^+ ATPaze u membranama ćelija krvi nakon izlaganja kompleksima zlata. Rezultati su prikazani na sledećim slikama (16-21).



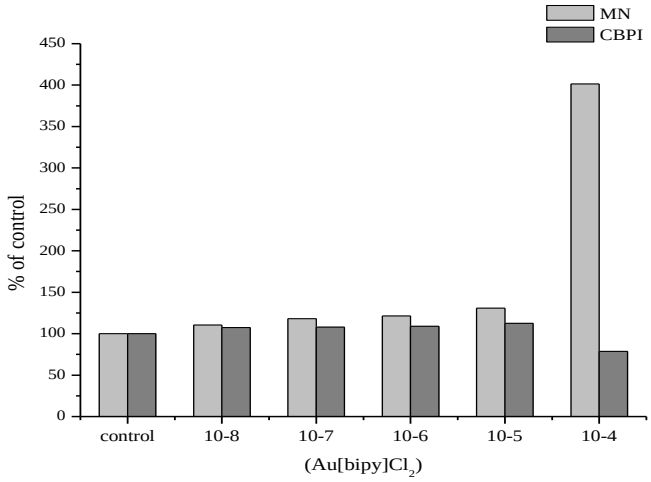
Slika 16. - Rezultati mikronukleusnog i proliferacionog testa za $[AuCl_4]^-$ jon.

$[AuCl_4]^-$ jon pokazuje slab genoprotektivni efekat u koncentracijama iznad 10^{-7} M, jer sprečava nastanak mikronukleusa, kao što se vidi na slici 16. Potencijal ćelija ka proliferaciji nije značajno promenjen ili je u blagom porastu. Iz prikazanog rezultata je moguće zaključiti da $[AuCl_4]^-$ nema citotoksičan efekat.



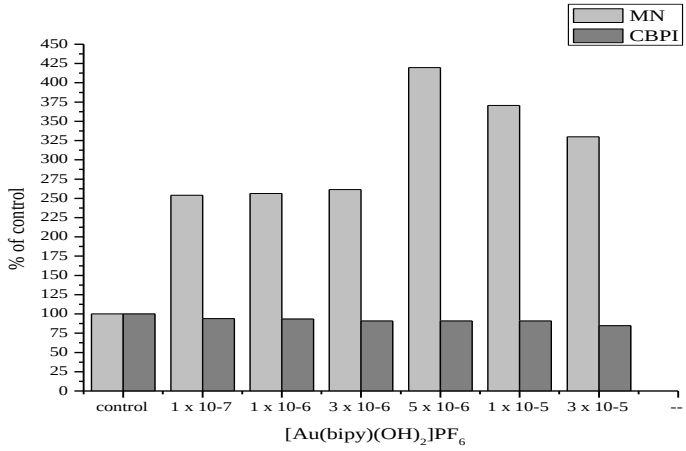
Slika 17. - Rezultati mikronukleusnog i proliferacionog testa za AuDMSO.

AuDMSO, kao i $[AuCl_4]^-$, ne pokazuje sklonost ka izazivanju porasta učestalosti mikronukleusa. Naprotiv, kao i $[AuCl_4]^-$, i AuDMSO dovodi do smanjenja broja uočenih mikronukleusa, koji je praćen neizmenjenim ili blago povećanim proliferacionim indeksom, kao što se vidi na slici 17.



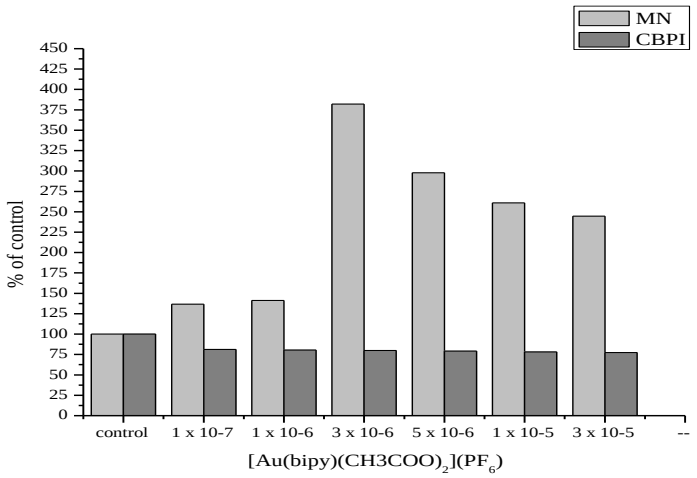
Slika 18. - Rezultati mikronukleusnog i proliferacionog testa za Aubipy.

Aubipy pokazuje jasan genotoksični efekat zavisan od doze. Sa porastom koncentracije raste broj uočenih mikronukleusa i opada proliferacioni indeks limfocita, kao što je pokazano na slici 18. Ovi efekti postaju dramatični pri koncentracijama preko 10^{-5} M.



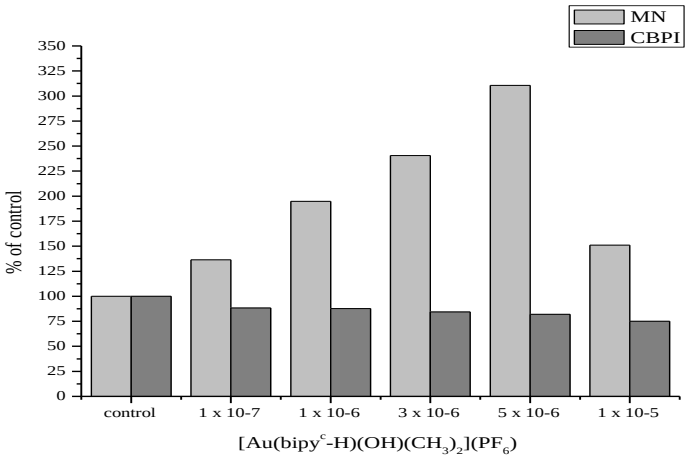
Slika 19. - Rezultati mikronukleusnog i proliferacionog testa za AubipyOH.

AubipyOH pokazao je još veći citotoksični potencijal od Aubipy. Broj mikronukleusa je i pri najnižim koncentracijama za više nego duplo prevazišao kontrolni uzorak, što pokazuje slika 19. Proliferacioni indeks opada u skladu sa porastom koncentracije, što jasno pokazuje da ovaj kompleks poseduje izuzetnu citotoksičnost. Iznad koncentracije od 10^{-6} M pretežno dolazi do smrti cele ćelije i ne pojavljuju se novi mikronukleusi, pa je njihov broj na grafiku prividno smanjen.



Slika 20. - Rezultati mikronukleusnog i proliferacionog testa za Aupy.

Premda ne jednako agresivan kao AubipyOH, Aupy također je pokazao jak citotoksičan učinak, što se vidi na slici 20. Velika učestalost mikronukleusa i opadanje proliferacionog potencijala prate porast doze, ali postaju upečatljivi tek pri koncentracijama oko 10^{-6} M, dok iznad te koncentracije, kao i u slučaju AubipyOH, citotoksični efekat postaje suviše jak i ne formiraju se novi mikronukleusi već ćelije odumiru.

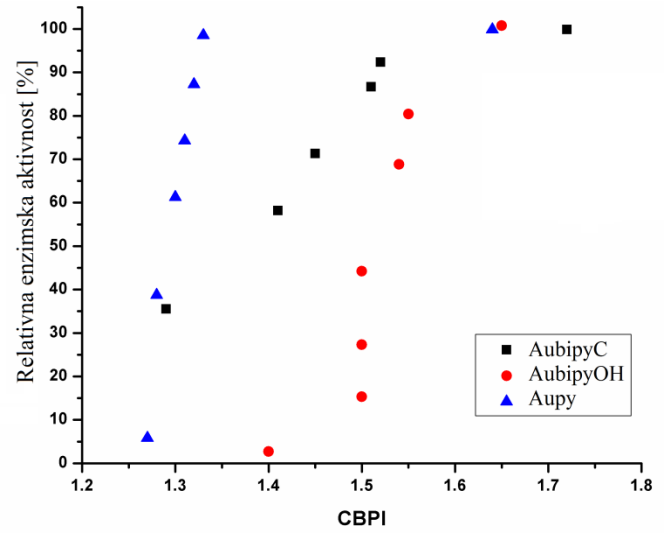


Slika 21. - Rezultati mikronukleusnog i proliferacionog testa za AubipyC.

AubipyC pokazuje visoku citotoksičnost i takođe dovodi do dozno zavisnog porasta broja mikronukleusa i opadanja proliferacionog indeksa, kao što je prikazano na slici 21. U ovom slučaju uočava se visok stepen korelacije između porasta koncentracije kompleksa i promena u broju mikronukleusa i proliferacionom potencijalu. Pri visokim koncentracijama (oko 10^{-5} M) AubipyC ispoljava jako citotoksično delovanje koje pre dovodi do smrti ćelije nego do pojave mikronukleusa, kao što je bio slučaj kod kompleksa AubipyOH i Aupy.

4.2.7. Korelacija između citotoksičnosti i aktivnosti enzima

Za komplekse koji su pokazali značajnu citotoksičnost (AubipyC, Aupy i AubipyOH) utvrđeno je postojanje korelacije između stepena inhibicije Na^+/K^+ ATPaze i proliferacionog indeksa. Ova korelacija prikazana je na slici 22.



Slika 22. – Korelacija između stepena inhibicije Na^+/K^+ ATPaze u membrane ćelija i proliferacionog indeksa.

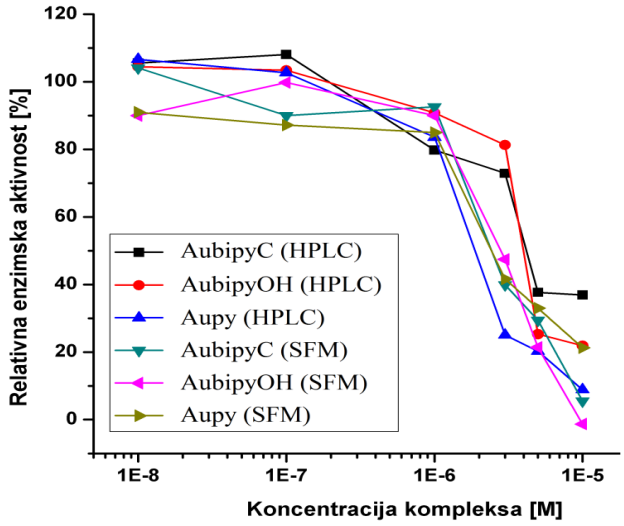
Uočava se da sa porastom aktivnosti enzima (pri nekoj koncentraciji inhibitora) raste i indeks proliferacije ispitivanih ćelija (pri istoj koncentraciji inhibitora). Ova korelacija najizraženija je za kompleks AubipyC

4.2.8. Određivanje aktivnosti enzima u uzorcima krvi

Aktivnost enzima merena je i u uzorcima dobijenim odvajanjem membrana ćelija humane krvi, kako bi se proverilo ponašanje kompleksa u simuliranim *in vivo* uslovima. Nakon

tretiranja ćelija kompleksima, njihove membrane su odvojene prema opisanoj metodi i u njima je merena aktivnost Na^+/K^+ ATPaze uz upotrebu standardnog testa. U pojedinim slučajevima, kada je korišćena spektrofotometrijska metoda za merenje količine oslobođenog fosfata, dobijeni su abnormalni rezultati, pa se pribeglo upotrebi HPLC metode za merenje odnosa ADP i ATP, kako bi se proverila spektrofotometrijska metoda.

Na slici 23. je prikazano poređenje rezultata dobijenih preko HPLC metode za praćenje odnosa ATP i ADP i spektrofotometrijske metode za određivanje fosfata.

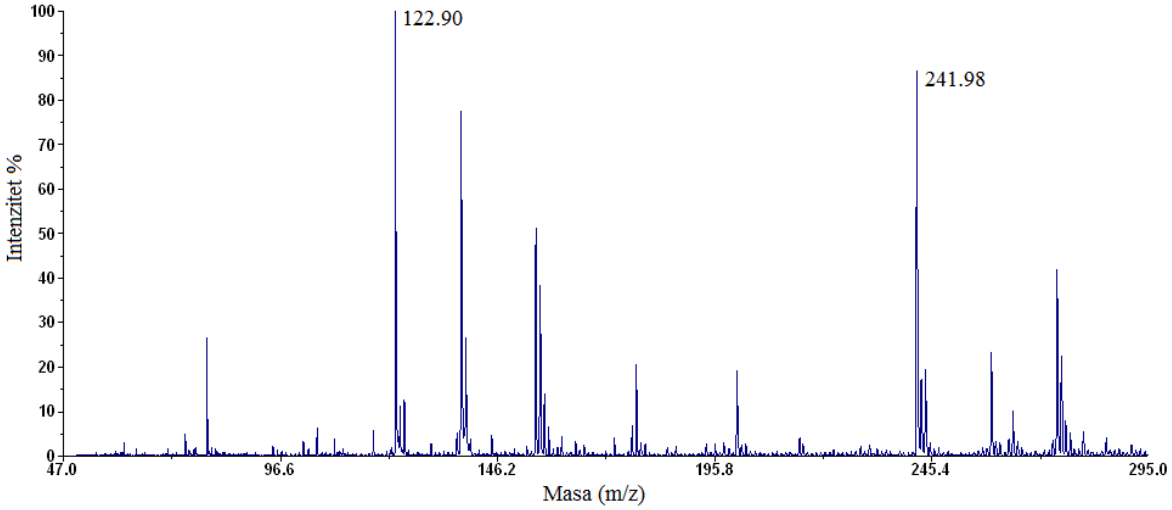


Slika 23. – Aktivnost enzima u uzorku membrana humanih ćelija krvi, poređenje HPLC metode (HPLC) i spektrofotometrijske (SFM) metode

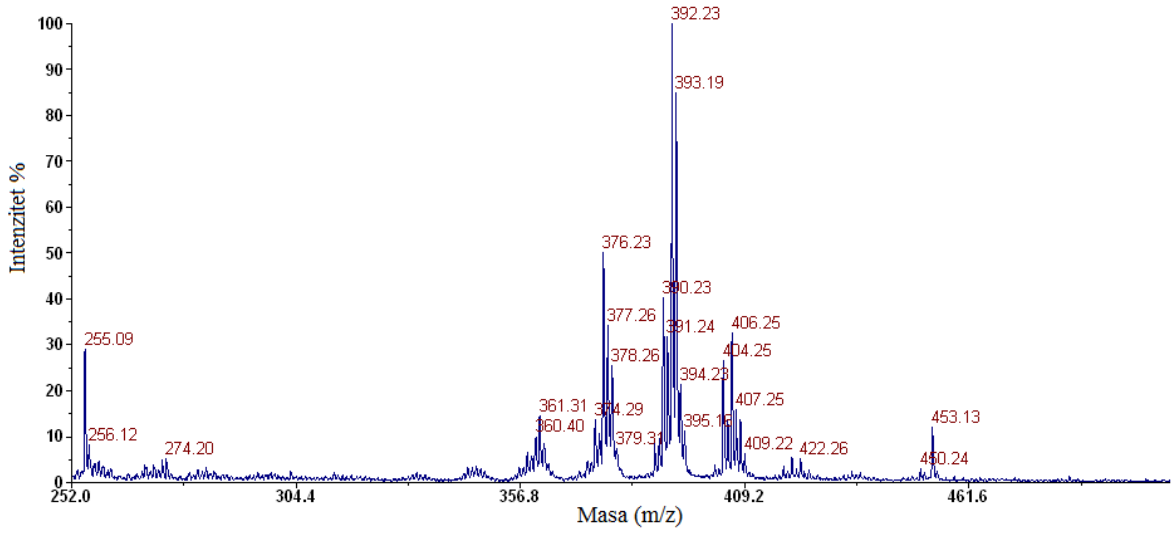
Uočava se da je saglasnost između dve metode velika. Obe metode pokazuju očigledan trend opadanja enzimske aktivnosti sa porastom primenjene količine kompleksa. Poređenjem sa grafikom enzimske inhibicije uočava se saglasnost između vrednosti IC_{50}.

4.2.9. Analiza kompleksa zlata masenom spektrometrijom

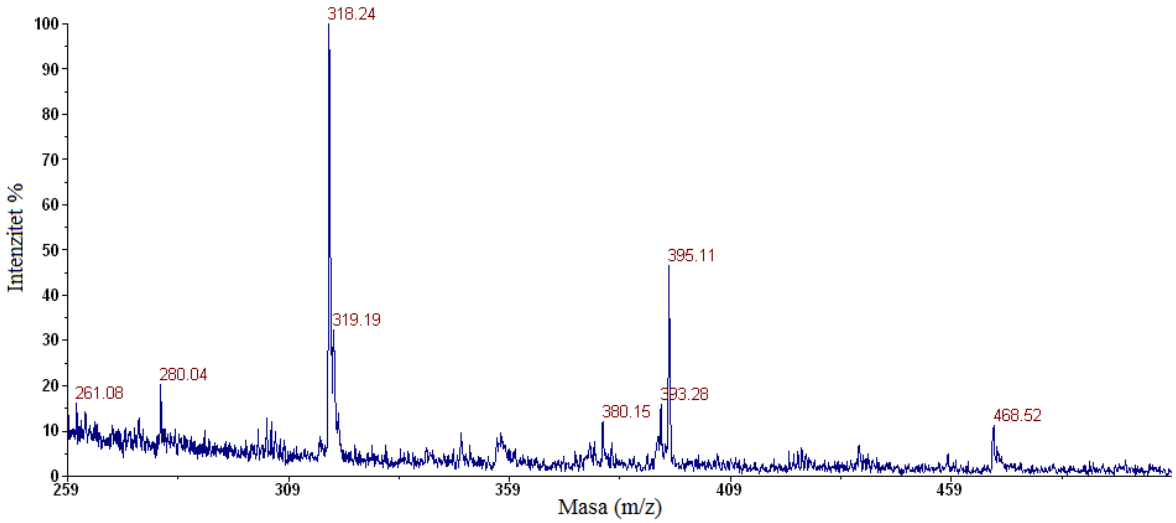
Radi konačne potvrde interakcije ispitivanih kompleksa zlata sa –SH grupama enzima i malih molekula napravljen je set snimaka na MALDI TOF MS sistemu za uzorke cisteina (u obliku hidrohlorida), kompleksa AubipyOH i njihove smeše u odnosu 10^{-4} M kompleksa prema 10^{-3} M cisteina. Snimljeni spektri su prikazani na slikama 24, 25 i 26.



Slika 24. – Maseni spektar uzorka cistein hidrohlorida.



Slika 25. – Maseni spektar uzorka AubipyOH.

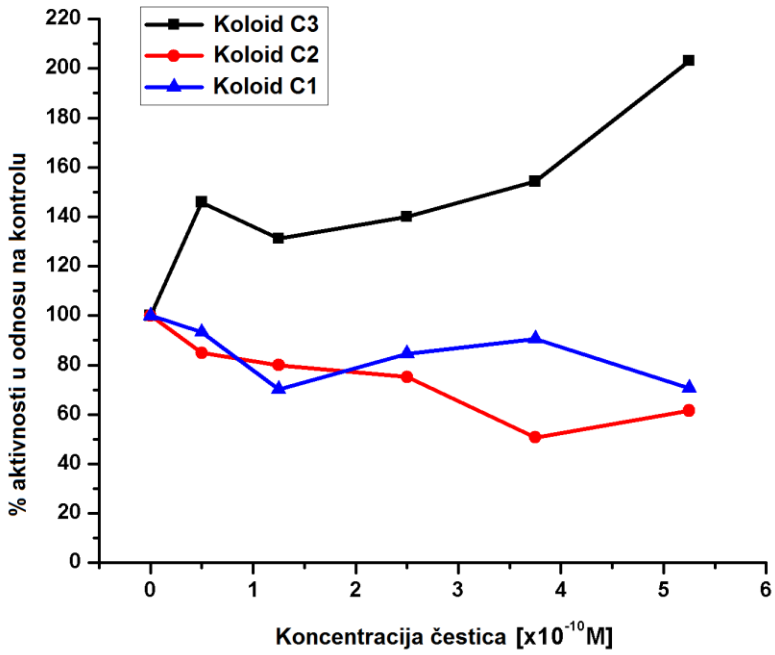


Slika 26. – Maseni spektar smeše AubipyOH i cistein hidrohlorida u odnosu 10^{-4} M kompleksa prema 10^{-3} M cisteina.

4.3. Nanočestice zlata

4.3.1. Uticaj nanočestica zlata na sinaptozomalne plazma membrane

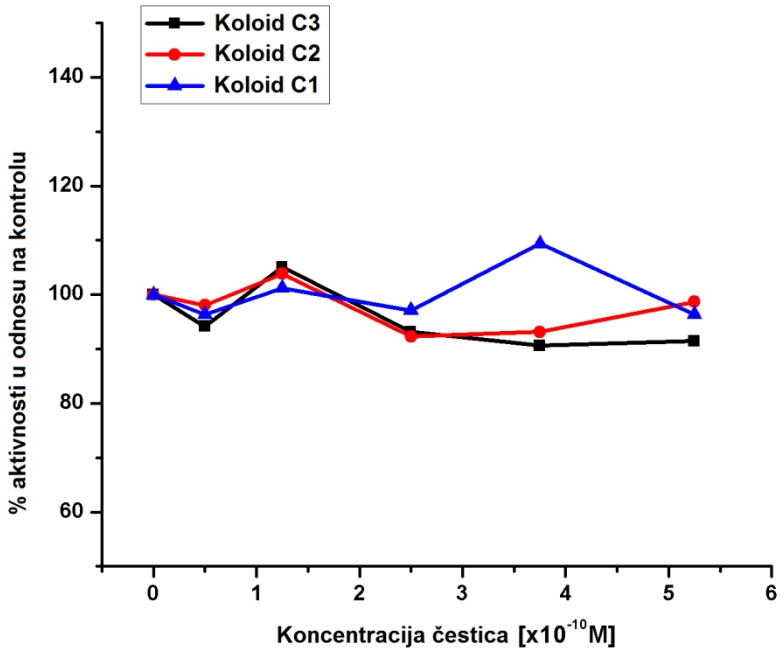
Početna hipoteza ovog eksperimenta je bila da će interakcija koloidnih čestica zlata i preparata SPM dovesti do pada aktivnosti Na^+/K^+ ATPaze usled vezivanja enzima za jone i atome zlata. Ova hipoteza je eksperimentalno opovrgnuta, čime se stvorio prostor za dublju analizu prirode interakcije između koloida i preparata SPM. Ispitivanja su vršena tako što je u enzimski test dodavana određena koncentracija koloida i potom je na istovetan način kako je gore opisano merena aktivnost enzima Na^+/K^+ ATPaze. Inhibicija je, međutim, izostala u slučaju koloida C1 i C2. U slučajevima kada je dodavan koloid C3 u enzimski test, dolazilo je do povećanja relativne aktivnosti enzima na način zavisan od koncentracije. Rezultati su prikazani na slici 27.



Slika 27. – Zavisnost aktivnosti Na^+/K^+ ATPaze u preparatu SPM od koncentracije nanočestica koloida C3.

Uočava se izuzetno povećanje relativne aktivnosti enzima tretiranog koloidom C3, koja pri višim koncentracijama koloida prevazilazi 200%. C1 i C2 nisu bitno menjali aktivnost enzima.

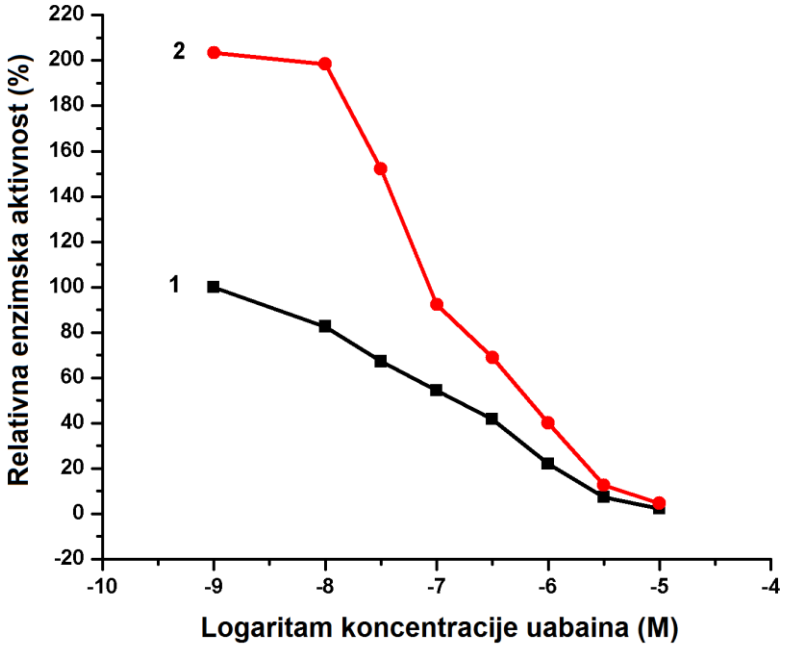
Pošto je aktivnost Na^+/K^+ ATPaze praćena na modelu SPM, konstantno je praćena i aktivnost pratećih ATPaza iz uzorka, gde nije uočena veća promena u aktivnosti. Rezultat je dat na slici 28.



Slika 28. – Zavisnost aktivnosti pratećih ATPaza u preparatu SPM od koncentracije nanočestica koloida C3.

Utvrđeno je da se najveće povećanje aktivnosti dobija pri odnosu od 25 μg proteina (kao SPM) i 7,33 × 10^{-10} M nanočestica koloida C3. Tada relativna aktivnost enzima iznosi (247 ± 5) % u odnosu na kontrolu. Nakon ovog odnosa, dalje dodavanje nanočestica dovodi do opadanja aktivnosti enzima.

Bilo je potrebno ispitati očuvanost selektivnosti enzima za njegove prirodne inhibitore, pa je ispitan uticaj uabaina na imobilizovani enzim. Postavljena su dva paralelna enzimska testa, s tom razlikom što je u jedan dodavano 100 μL koloida C3 umesto 100 μL vode. U oba testa dodavan je inhibitor uabain u prikazanim koncentacijama. Rezultati su prikazani na slici 29.

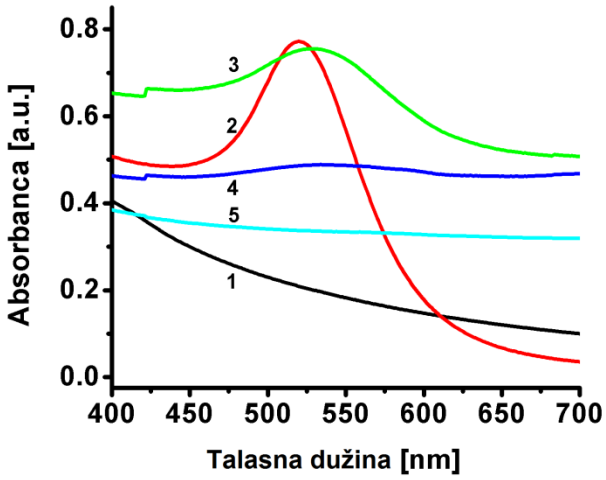


Slika 29. – Inhibicija netretiranog (1) i tretiranog (2) preparata SPM uabainom.

Uočava se da je IC_{50} za uabain iste vrednosti, kao i da je proba u kojoj se nalazi koloid C3 osetljivija.

4.3.2. Priroda interakcije nanočestice i SPM

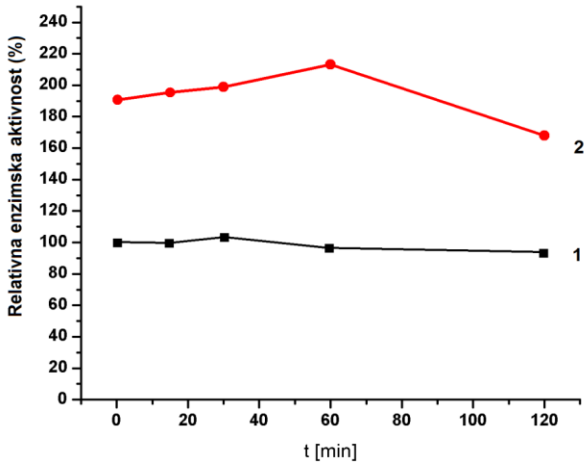
U toku prvih merenja promene aktivnosti po dodatku nanočestica u preparat SPM uočeno je nekoliko fenomena koji su ukazali na mogući mehanizam te interakcije. Kako prečišćen enzim nije povećao aktivnost po dodatku nanočestica i kako –SH donori nisu remetili povećanje koje je uočeno po dodatku C3 u uzorak SPM, zaključeno je da je za interakciju koja dovodi do povećanja Na^+/K^+ ATPazne aktivnosti preparata SPM najverovatnije odgovorne membrane ćelija. Snimljeni su UV-vis spektri plazmonskog pika koloida C3 u odsustvu i prisustvu SPM tokom vremena. Uzorci su sadržavali 66,6 μg/mL SPM i $3,3 \times 10^{-11}$ M nanočestica zlata i snimani su tokom 20 minuta. Snimljeni pojedinačni spektri ovih komponenti su bili kontrolni uzorak. Dobijeni spektri su prikazani na slici 30.



Slika 30. – Promena spektra rastvora 3,3 × 10^{-11} M koloida C3 (2) po dodatku 66,6 μg/mL SPM (1) nakon 1 min (3), 10 min (4) i 20 min (5).

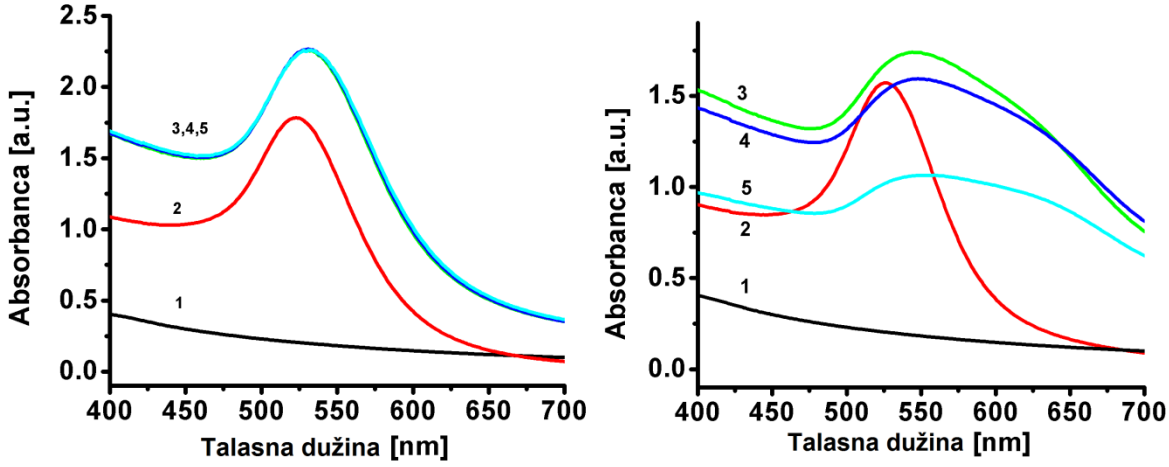
Vrlo jasno se uočava gubitak plazmonskog pika. Ovo je definitivan indikator uspešnosti pokrivanja nanočestica zlata fragmentima SPM.

Povećanje aktivnosti do kojeg dovodi koloid C3 nije zavisno od vremena preinkubacije u testu i čak i pri vrlo kratkoj preinkubaciji (< 1 min) ne izostaje efekat povećanja aktivnosti. Duža preinkubacija ne dovodi do jačeg efekta, što sugeriše da je reč o gotovo momentalnoj interakciji. Promena aktivnosti do koje dovodi C3 praćena je i u vremenu, a taj rezultat je prikazan na slici 31.



Slika 31. – Promena aktivnosti netretiranog (1) i tretiranog (2) enzima sa vremenom.

Uočava se da dolazi do opadanja aktivnosti tretiranog enzima nakon prvog sata, najverovatnije zbog fizičkog vezivanja enzima u talog i njegovog precipitiranja iz rastvora. Tokom prvog sata nema većih razlika u aktivnosti enzima i suspenzija je stabilna. Iako kod njih nije uočen efekat povećanja enzimske aktivnosti, snimljeni su spektri koloida C1 i C2 pod istim uslovima kao i za koloid C3 gore. Rezultujući spektri dati su na slici 32.

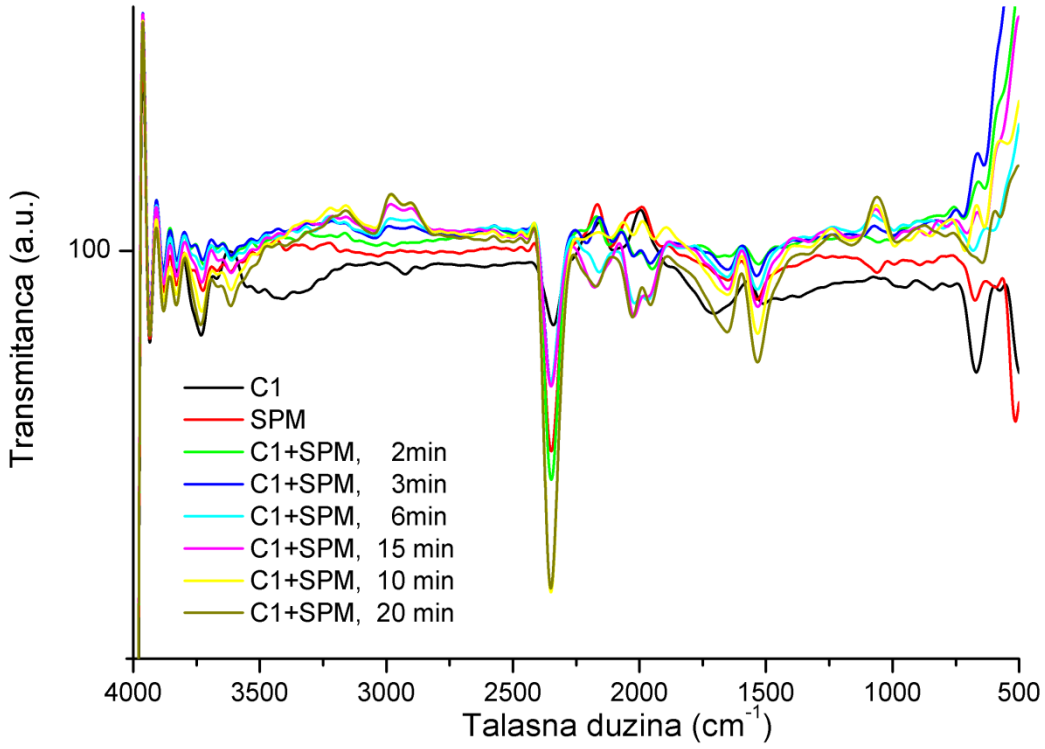


Slika 32. – **Levo:** Promena spektra rastvora 3,3 × 10^{-11} M koloida C2 (2) po dodatku 66,6 μg/mL SPM (1) nakon 1 min (3), 10 min (4) i 20 min (5). **Desno:** Promena spektra rastvora 3,3 × 10^{-11} M koloida C1 (2) po dodatku 66,6 μg/mL SPM (1) nakon 1 min (3), 10 min (4) i 20 min (5).

U spektru koloida C2 se ne uočavaju gotovo nikakve promene izuzev blagog pomeranja plazmonskog pika u desno, dok u spektru koloida C1 dolazi do deformacije plazmonskog pika.

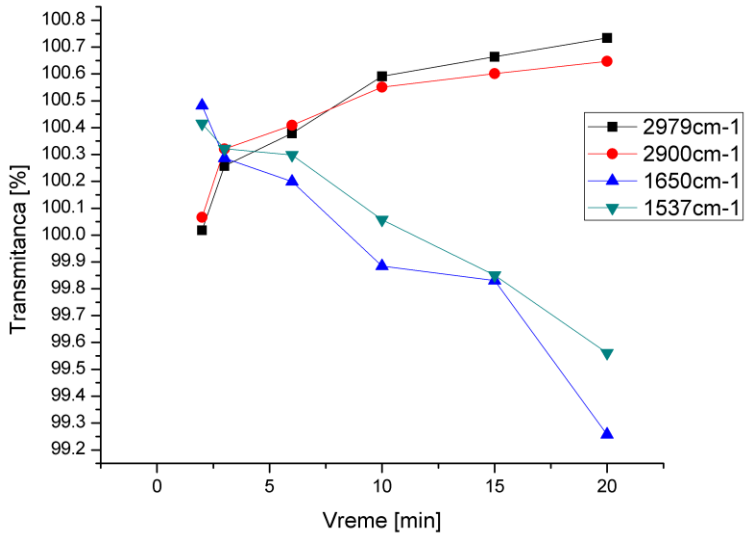
4.3.3. Praćenje interakcije membrane i nanočestice infracrvenom spektroskopijom

Radi razjašnjavanja mehanizma procesa imobilizacije SPM na površini koloida, pristupilo se snimanju FTIR spektara, sa namerom da se uoče promene u karakterističnim pikovima. Rastvori sva tri koloida su snimljeni. Dobijeni spektri dati su na slikama 33, 35 i 37. Trake između 2250 i 2500 cm^{-1} su artefakti koji potiču od CO_2.

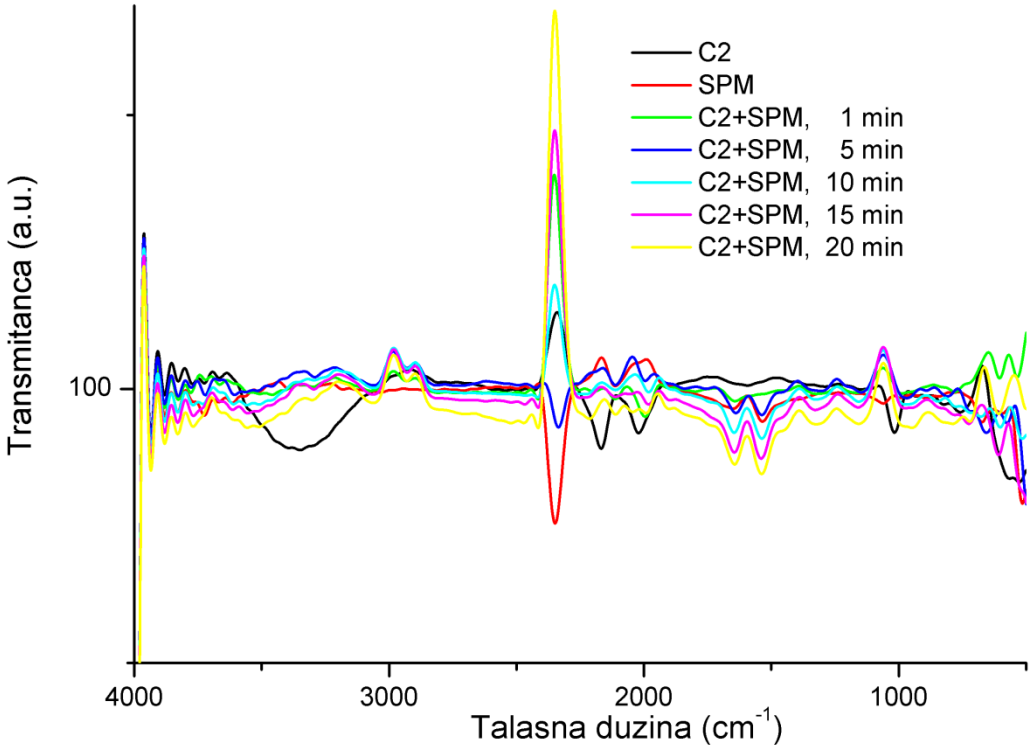


Slika 33. – FTIR spektri SPM, koloida C1 i njihove smeše tokom 20 minuta.

Na spektru se jasno vide dve populacije pikova čiji se oblik i intenzitet menjaju sa vremenom. To su grupe pikova koji se nalaze od 1500 do 1700 cm^{-1} i od 2800 do 3000 cm^{-1} u spektru. Njihova promena kroz vreme prikazana je na sledećem grafiku i daje uvid u dinamiku ove interakcije.

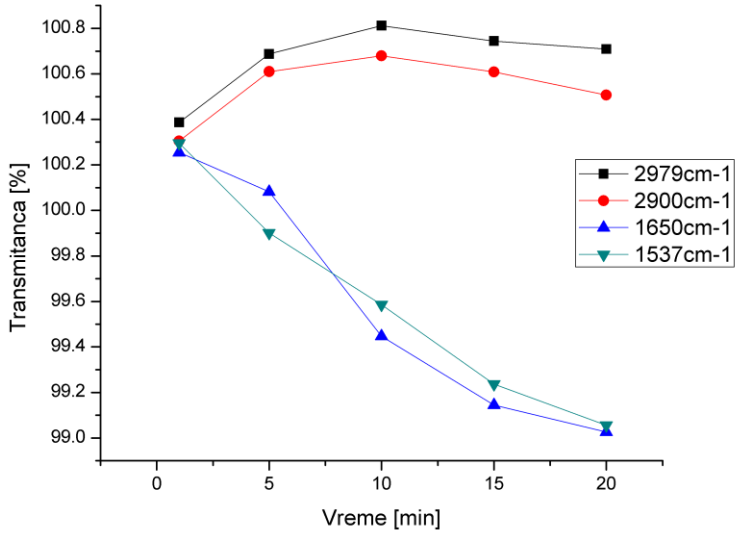


Slika 34. – Promene izdvojenih pikova za C1 tokom vremena.



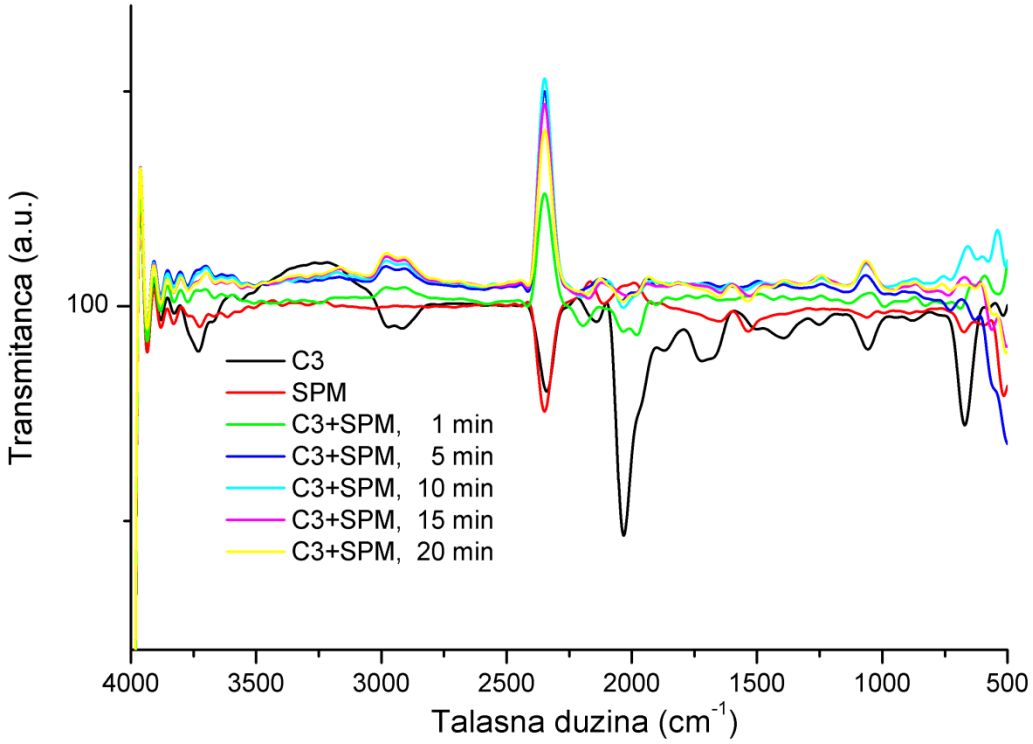
Slika 35. - FTIR spektri SPM, koloida C2 i njihove smeše tokom 20 minuta.

U slučaju koloida C2 takođe se uočavaju grupe pikova od 1500 do 1700 cm^{-1} i od 2800 do 3000 cm^{-1}. I ovog puta vrednosti za ove pikove izdvojene su i prikazane na odvojenom grafiku u funkciji vremena.

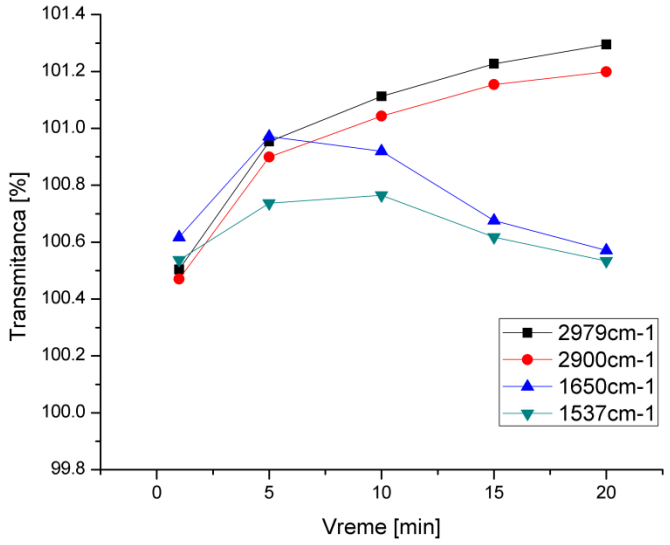


Slika 36. - Promene izdvojenih pikova za C2 tokom vremena.

Koloid C3, koji je dao efekat povećanja aktivnosti enzima na preparatu takođe je snimljen na FTIR spektrometru. Dobijeni spektar prikazan je na slici 37. I u ovom spektru uočavaju se promene u regionu od 2800 do 3000 cm^{-1}, ali izostaju promene u regionu oko 1500 cm^{-1}.



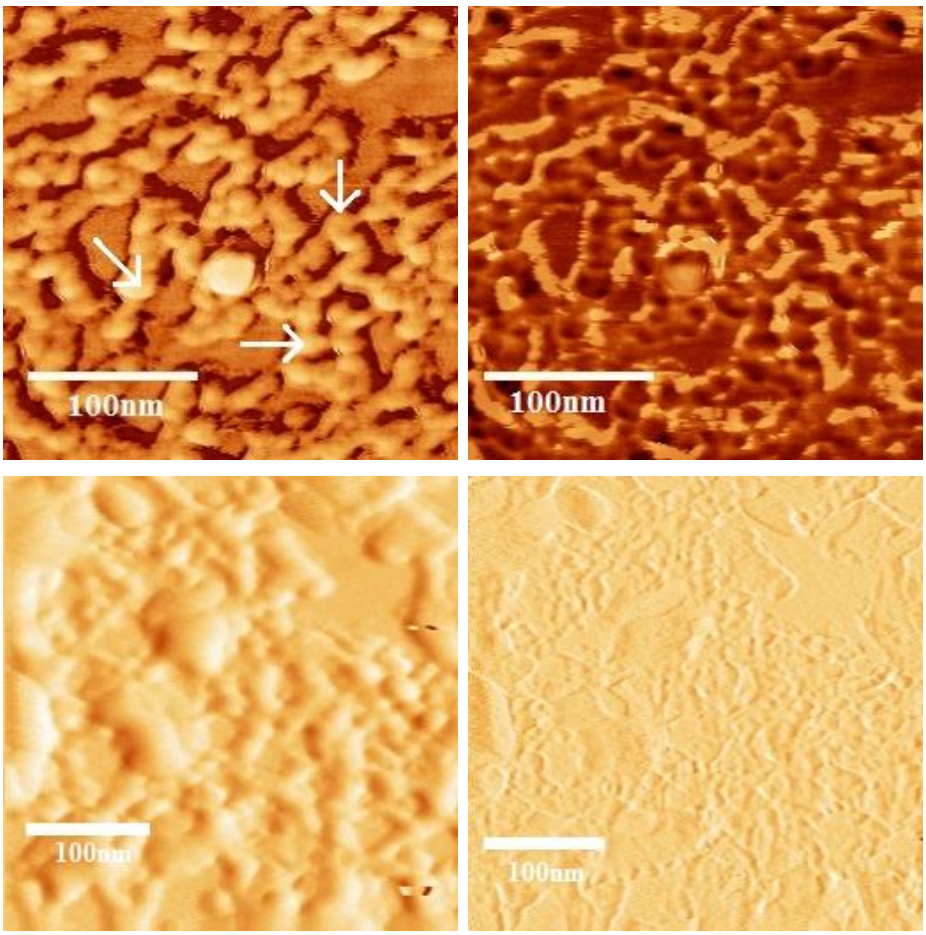
Slika 37. - FTIR spektri SPM, koloida C3 i njihove smeše tokom 20 minuta.



Slika 38. - Promene izdvojenih pikova za C3 tokom vremena.

4.3.4. Atomske mikrografije nanočestica i sinaptozomalnih plazma membrana

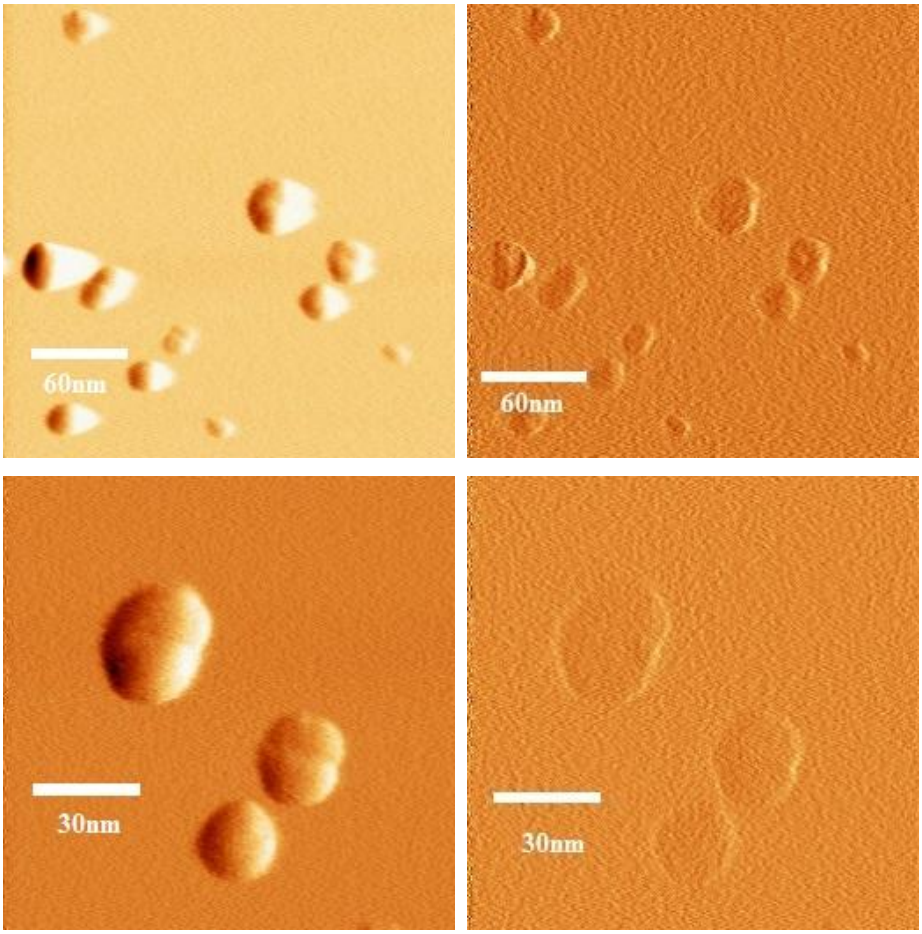
Sa namerom da se uoči interakcija koja je ovde opisana napravljene su AFM mikrografije koloida i SMP, kao i njihovih smeša na način koji je opisan u eksperimentalnom delu.



Slika 39. – AFM snimak netretiranog SPM materijala. Levo je fazni prikaz, desno topografski, ista oblast. Dole isto, drugi uzorak. Bele strelice pokazuju zadebljanja usled prisustva proteina.

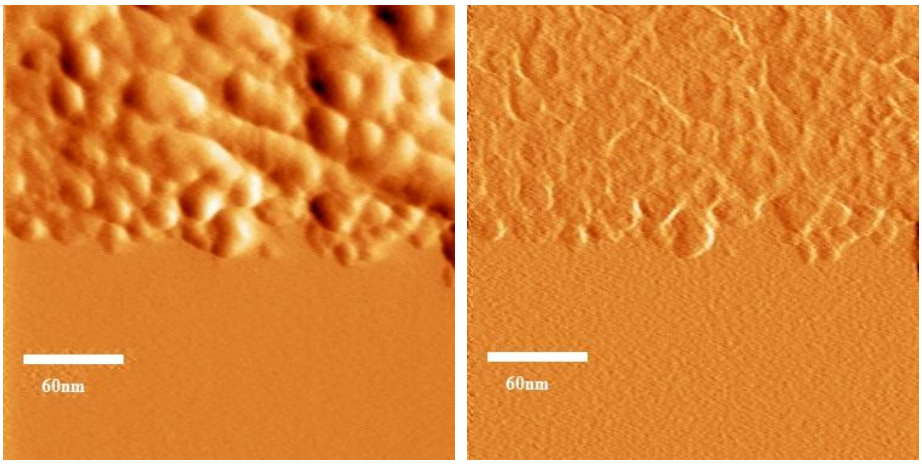
Na snimku materijala SPM uočava se vrpčasta struktura slepljenih membrana. Zadebljanja koja se uočavaju na snimku (označena strelicama) su proteini, proteinski agregati ili membranski agregati, koji su nastali tokom pripreme i dobijanja SPM, verovatno tokom agresivnog i dugotrajnog ultracentrifugiranja koje je opisano u poglavlju o pripremi SPM. Deo enzima Na^+/K^+ ATPaze zarobljen je u tim agregatima, slepljen sa membranama i nije dostupan za reakciju. Materijal je heterogen i ne uočavaju se ponavljanja u strukturi koja bi bila karakteristična za homogen materijal. Pri snimanju materijala sa manjom visinom igle, materijal se "lepi" za

iglu i javljaju se artefakti vidljivi na slici 40 gore levo. Na faznom prikazu se uočavaju "grudvice" unutar materijala koje po dimenzijama (15 - 20 nm) odgovaraju i najverovatnije jesu enzim Na^+/K^+ ATPaze (~15 nm), ali mogu poticati i od nekih drugih velikih molekulskih kompleksa u membrani ili nečistoća.



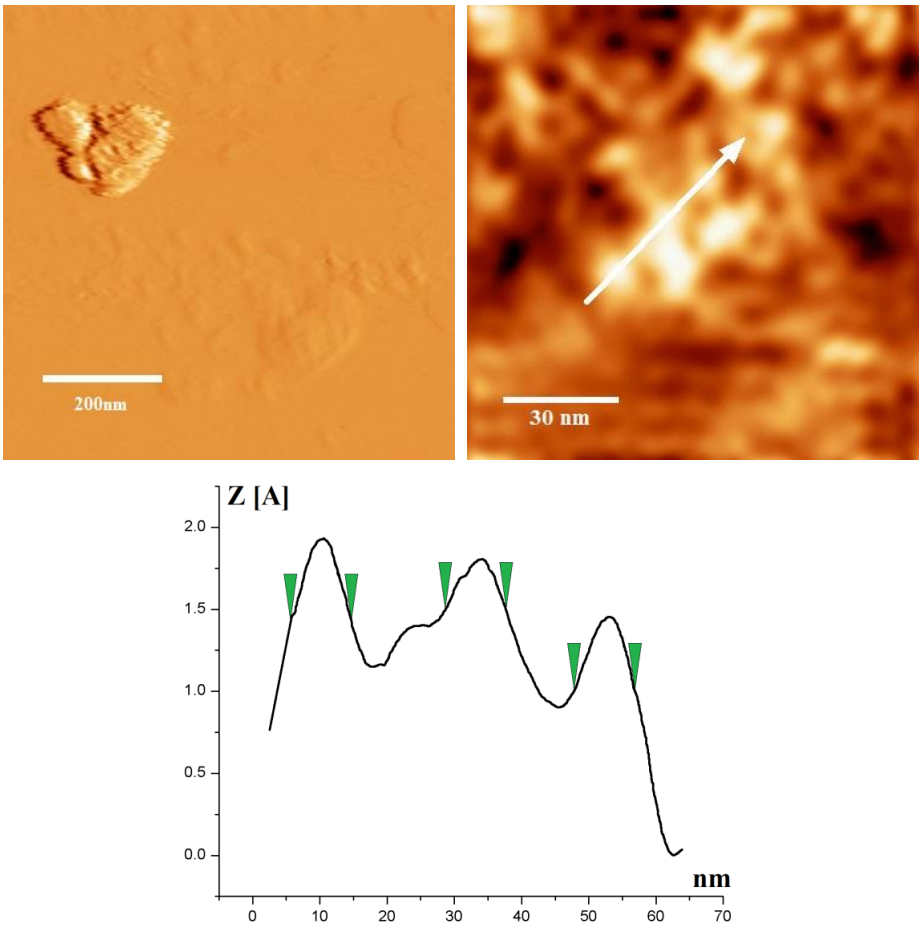
Slika 40. – Nanočestice koloida C1 u faznom (levo) i topografskom (desno) modu snimanja. Jasno su uočljive sferne nanočestice dimenzija oko 30 nm. Dole isto, uvećano.

Nanočestice koloida C1 su vidljive na AFM mikrografijama i pokazuju veliki stepen dispergovanosti na podlozi. Pretežno su pravilnog, sfernog oblika, ređe jajaste ili slepljene. Pri nižoj visini igle za snimanje lako se kotrljaju po HOPG površini. Najveću populaciju predstavljaju čestice dimenzija oko 30 nm, ali prisutne su i manje čestice. Površina čestice je glatka, izuzev u slučaju slepljenih čestica gde postoji ugibljenje, kao što se vidi na mikrografijama na slici 41.



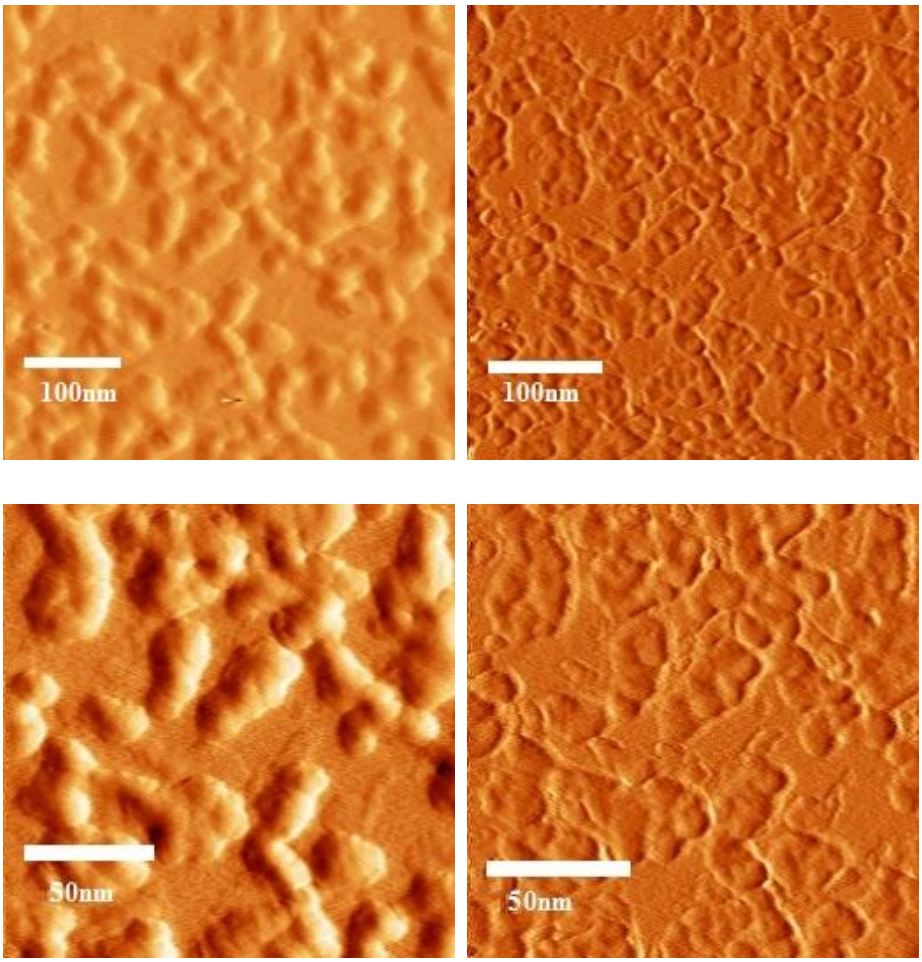
Slika 41. - Nanočestice koloida C2 u faznom (levo) i topografskom (desno) modu snimanja. Čestice su slepljene i nisu jasno vidljive pojedinačne čestice.

Nanočestice koloida C2 tokom pripreme za snimanje na HOPG podlozi agregiraju u "grozdove" ili klastere ali ostaju vidljive. Kako do ove pojave ne dolazi u radnom rastvoru, hipoteza je da je ovaj tip čestica nestabilan i da u interakciji sa podlogom tokom sušenja biva destabilisan što dovodi do slepljenih agregata poput prikazanih. Snimanja mešavine koloida C2 i SPM su urađena, ali dobijene slike su neinformativne i nemoguće ih je tumačiti, jer se na njima ne uočava da dolazi do bilo kakve jasno vidljive interakcije SPM i nanočestica. Nije jasno da li je to posledica karakteristika nanočestica ili pripreme uzoraka za AFM, ali na osnovu ranije prikazanih rezultata odgovor najverovatnije leži u karakteristikama same nanočestice, jer je izostao biološki efekat, kao i promena u UV-Vis spektrima mešavine koloida C2 i SPM.



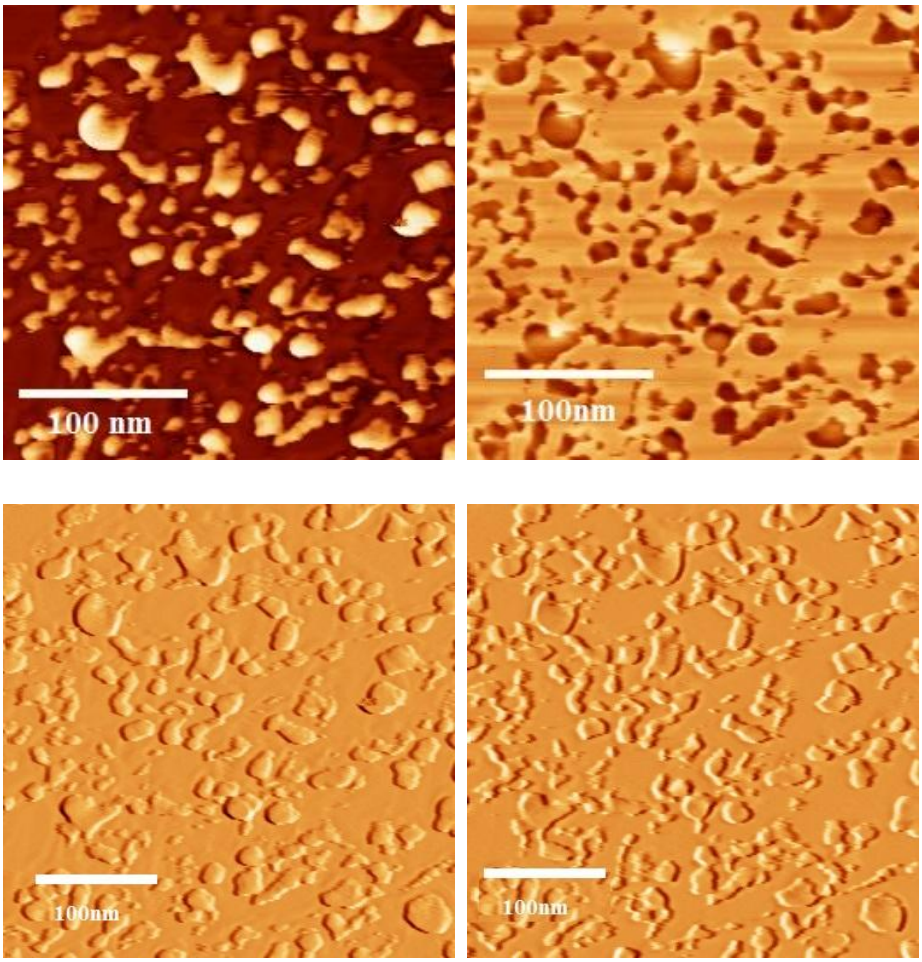
Slika 42. - Nanočestice koloida C3 u faznom modu snimanja (gore levo) uvećano (gore desno) i izvučeni visinski profil iz snimka (dole).

Glavni problem tokom snimanja nanočestica koloida C3 bila je njihova mala dimenzija, koja je blizu donje granice detekcije korišćenog uređaja pod korišćenim uslovima snimanja. Slično koloidu C2, koloid C3 je tokom pripreme za snimanje i sušenja rastvora težio da formira klasterske strukture, poput ove prikazane na slici 42 gore levo. Ovi klasteri bili su manji nego kod C2. Na grafiku je prikazan profil napravljen po strelici na mikrografiji, odakle se vidi da dimenzija čestica odgovara ranije publikovanim dimenzijama ovih čestica, od 9,5 do 10 nm.



Slika 43. – Smeša koloida C1 i SPM u faznom (levo) i topografskom (desno) modu. Donji red isto, uvećano.

Na snimcima mešavine koloida C1 i SPM vidi se da sinaptozomalne membrane dovode do slepljivanja nanočestica. Snimci ukazuju na to da nastaju agregati sastavljeni od SPM i nanočestica u kojima je kontaktna površina membrane sa rastvorom smanjena. Pojedinačne nanočestice se ne uočavaju, a ne uočavaju se ni neravnine na površini ćelijskih membrana, što sugeriše da je došlo do slepljivanja pre nego do oblaganja nanočestica membranama. Nastali agregati se stajanjem ujedinjuju u veće agregate, sve dok ne postanu makroskopski vidljivi i počnu da precipitiraju, kao što je uočeno tokom snimanja UV-vis i FTIR spektara. Usled ovoga izostaje efekat povećanja aktivnosti enzima u membrani.



Slika 44. - Smeša koloida C3 i SPM u faznom (levo) i topografskom (desno) modu. Dole isto, drugi uzorak.

Kada se uporede AFM snimci SPM i ovde prikazani snimci smeše SPM i nanočestica koloida C3 više je nego očigledno da su komadići membrane usitnjeniji i da je aktivna površina ćelijskih membrana daleko veća. Membranski fragmenti su izdeljeni na male "kapljice", njihova relativna visina je mala u odnosu na HOPG podlogu, za razliku od čistih SPM koji su u velikim agregatima, lancima ili "grudvama". Nastali fragmenti su diskretni i ne slepljuju se. Ne uočavaju se pojedinačne nanočestice, ali se iz dimenzija nastalih konglomerata nanočestica i membrana može zaključiti da u jednom komadiću membrane može biti prisutno nekoliko nanočestica. U tom smislu ne može se govoriti o pravoj imobilizaciji na površini nanočestice, već više o oblaganju nanočestice ili grupe nanočestica fragmentima SPM. Posledica tog oblaganja i urođene težnje membrane da smanji kontaktnu

površinu sa vodenom sredinom jeste formiranje veoma usitnjenih agregata, možda i micela, koji imaju veliku aktivnu površinu i time dovode do prividnog povećanja enzimske aktivnosti Na^+/K^+ ATPaze.

5. Diskusija

Rezultati inhibicionih testova pokazali su da je od svih ispitivanih kompleksa najjači inhibitor kompleks $[AuCl_4]^-$. Ovo ne iznenađuje, jer je kompleks $[AuCl_4]^-$ najreaktivniji, zbog malih i lako izmenljivih liganada. Efekat inhibicije trifosfataza ovim kompleksom poznat je iz ranije literature, ali tada nije dublje proučavan [43]. S druge strane, dinuklearni i koronarni kompleksi Au-oxo6, Au2phen i Aucyclam pokazuju veliku inertnost prema enzimu, najverovatnije usled njihove hidrofobnosti i visoke stabilnosti. Oni nisu pokazali inhibitorni efekat. Centralni jon je dobro zaštićen i nije dostupan za vezivanje sa enzimom. Hilovom analizom je pokazano da postoji pozitivna kooperativnost u vezivanju molekula inhibitora. Uz pretpostavku da se vezivanje odvija preko –SH grupa, postoji preko 60 potencijalnih vezivnih mesta. Najverovatnije se, međutim, vezivanje ne događa na svim mestima. Pozitivna kooperacija upućuje na zaključak da dolazi do strukturnih promena u enzimu koje čine druga vezivna mesta dostupnijim, verovatno usled denaturacije ili oksidacije.

Kada je reč o uticaju na sporedne ATPaze, kao što se može i očekivati, najveći pad aktivnosti sporednih ATPaza izazvao je kompleks $[AuCl_4]^-$. Ovo sugeriše da ispitivani kompleksi zlata mogu imati i druge potencijalne upotrebe, tj druge biološke molekulske mete. Kako mnogi enzimi u organizmu poseduju slobodne –SH grupe, opravdano je pretpostaviti da bi kompleksi zlata mogli da budu njihovi inhibitori, naročito ukoliko se te –SH grupe nalaze u regionu koji je od značaja za enzimsku funkciju.

Na osnovu dobijenih rezultata i dobijenih vrednosti srednjih inhibitornih koncentracija zaključuje se da su ispitivani kompleksi jaki inhibitori. Najveći afinitet pokazuje kompleks $[AuCl_4]^-$, tj tetrahloroauratni(III) jon, dok se slabija inhibicija javlja kod kompleksa sa velikim bipiridinskim ligandima. Uočavaju se i razlike u intenzitetu inhibicije u zavisnosti od toga koji ligandi su vezani kao mali i lako odlazeći ligandi. Kompleks Aupy ima acetatne grupe koje su lošiji odlazeći ligandi nego što je to slučaj kod kompleksa Aubipy i AubipyOH, dok kompleks AubipyC ima tridentatni ligand i samo jedan dobar odlazeći

ligand za vezivanje sa enzimom. Zbog toga kompleksi Aupy i AubipyC pokazuju najslabije inhibicije, premda su one i dalje relativno jake i ulaze u mikromolarni opseg.

U eksperimentima sa –SH donorima L – cisteinom i glutationom, –SH donori su uspeli da vežu za sebe deo kompleksa pre nego što je on izreagovao sa enzimom. Inhibicija koja se javlja, međutim, sugeriše da postoji određena hemijska ravnoteža i da proces nije u potpunosti kvantitativan, jer je –SH donor prisutan u mnogo puta većim koncentracijama od inhibitora, a ipak nije doveo do potpunog sprečavanja inhibicije. Moguće je i da postoje različite vrste proizvoda nastalih u reakciji između kompleksa i –SH donora, kao i da pojedini nastali proizvodi zadržavaju inhibitornu aktivnost. U slučajevima sprečavanja inhibicije ne uočava se neki naročit trend niti povećan afinitet pojedinih kompleksa ka vezivanju sa malim –SH donorom. Nešto jači preventivni efekat ima GSH, koji već u koncentraciji od 10^{-4} M daje značajan izostanak inhibicije. Ove vrednosti su značajne zato što odgovaraju vrednostima koje se nalaze u životinjskim ćelijama i mogu imati važan uticaj na ponašanje kompleksa *in vivo* [108]. U slučaju reaktivacije krive su više nalik hiperboli što sugeriše da postoje smetnje u procesu reaktivacije, najverovatnije poreklom od strukture enzima koja pretrpi promene po vezivanju kompleksa zlata i sterno otežava prilaz reaktivatoru. Moguće je da postoji i višestruko vezivanje nekoliko –SH grupa sa enzima za jedan molekul kompleksa, te dolazi do kooperativnog efekta koji otežava odlazak zlata. U literaturi postoje podaci o tome da kompleksi plemenitih metala mogu da dovedu do oksidativnog isecanja –S–S– mostova u proteinu i posledičnu ireverzibilnu inhibiciju, što bi moglo da objasni nepotpunu reaktivaciju enzima [109-112]. Tačan uzrok nepotpune reaktivacije enzima nije poznat. Za razliku od eksperimenata sa prevecijom inhibicije, ovde se ne javlja razlika u potrebnoj koncentraciji –SH donora da bi došlo do umanjenja inhibitornog efekta, ali se uočava da GSH pri višim koncentracijama inhibitora dovodi do višeg stepena reaktivacije nego što je to slučaj sa cisteinom. GSH uspostavlja preko 50% aktivnosti enzima, dok cistein uspostavlja samo 40%. Rezultati testova za sprečavanje inhibicije i reaktivaciju inhibiranog enzima nedvosmisleno ukazuju na veliki značaj –SH grupe u mehanizmu ostvarivanja enzimske inhibicije. Veći učinak redukovanog glutationa može se objasniti njegovom evolutivnom predodređenošću za stupanje u reakcije u

metabolizmu preko –SH grupe koju nosi [113]. Njegova –SH gupa je manje sterno zaštićena i struktura samog molekula favorizuje reakcije preko te grupe. Nedvosmisleno je da, ni sprečavanje inhibicije, ni reaktivacija enzima nisu potpune pri višim koncentracijama inhibitora. Takođe je jasno da proces vezivanja kompleksa za –SH donor nije kvantitativan i da se u reakcionom medijumu uspostavlja određena ravnoteža, što može implicirati delimičnu ireverzibilnu inhibiciju preko narušavanja geometrije proteina uslovljene –S-S-vezama, pojavu novih aktivnih vrsta kompleksa zlata i dodavanih –SH donora i/ili uključenost nekih drugih mehanizama u proces inhibicije, bilo putem interakcije zlata i njegovih kompleksa sa drugim aminokiselinskim ostacima, bilo preko postojanja eventualnih vezivnih mesta za kompleks koja ne uključuju interakciju sa jonom metala. Sve ovo otvara veliki prostor za dalja istraživanja mehanizma koji postoji u procesu vezivanja ispitivanih kompleksa za Na^+/K^+ ATPazu.

Kinetička merenja daju uvid u tip reverzibilne inhibicije. Svi ispitivani kompleksi pokazali su nekompetitivnu reverzibilnu inhibiciju. Ovo je u saglasnosti sa rezultatima iz literature koji postoje za slične komplekse i njihovo delovanje na Na^+/K^+ ATPazu [2-4, 44]. Konstante inhibicija, međutim, pokazuju određene nelogičnosti i ukazuju na mogućnost postojanja sporednih reakcija kompleksa zlata u vodenoj sredini. Najmanju konstantu inhibicije ima kompleks $[AuCl_4]^-$, kao što je i očekivano, pošto je on pokazao najjaču inhibiciju. Ali interesantno je da kompleks AuDMSO pokazuje najveću konstantu inhibicije iako se u ispitivanjima pokazao kao veoma jak inhibitor. Ovo može upućivati na to da ovaj kompleks podleže nekoj sporednoj reakciji u reakcionom medijumu zbog koje dolazi do nastajanja neke reaktivnije vrste kompleksa, ali u manjoj količini. Postoji vrlo malo podataka u literaturi koji se tiču ponašanja ovog kompleksa u vodenoj sredini, pa je ovo jedan od potencijalnih pravaca daljeg istraživanja [103]. Nešto slabiju konstantu inhibicije pokazuje i kompleks Aupy, najverovatnije usled prisustva lošijih odlazećih liganada acetata. Ne postoje druge značajne razlike između ostalih konstanti inhibicije i one su u saglasnosti sa ranije prikazanim rezultatima.

Eksperimenti na ćelijskim kulturama za neke od ovih kompleksa već su rađeni, te je tako lakše interpretirati dobijene rezultate. Naši rezultati su u dobroj saglasnosti sa rezultatima

sličnih studija. Naime, AubipyOH i AubipyC su do sada pokazali citotoksične efekte na nekoliko različitih tipova ćelija [8]. U našim eksperimentima, uočava se izostanak citotoksičnog efekta kod kompleksa $[AuCl_4]^-$ i AuDMSO, dok se jaki citotoksični efekti jasno vide kod ostalih ispitivanih kompleksa, koji sadrže bipiridinske ligande. Stoga se može zaključiti da njihova citotoksična aktivnost proizilazi upravo iz ovih liganada. Naime, bipiridinski ligandi i njihovi strukturno slični derivati poznati su po visokoj genotoksičnosti koja je posledica njihove aromatične strukture i sposovnosti da dovedu do oksidativnog oštećenja genetskog materijala. Njihova toksičnost iskazuje se i u kompleksima bipiridinskih derivata sa drugim metalima [114, 115]. Najverovatniji mehanizam delovanja podrazumevao bi funkciju planarnog liganda u asocijaciji sa DNK, koji bi potom pratila oksidativna oštećenja na DNK koja bi izazvala redukcija jona zlata. Ovaj mehanizam prisutan je kod analognih kompleksa platine i objasnio bi odsustvo naglašenog genotoksičnog delovanja kompleksa $[AuCl_4]^-$ i AuDMSO. Ovde je interesantno napomenuti da su dosadašnje studije za komplekse Au-oxo6, Au2phen i Aucyclam pokazale da oni poseduju citotoksična dejstva na različitim, često ispitivanim kulturama ćelija. Aucyclam se na sličan način kao i u ovde prikazanim rezultatima pokazao kao najmanje biološki aktivan, verovatno usled svoje izuzetno stabilne strukture i veze sa tetradentatnim cikličnim ligandom. Navedene studije pokazale su da kompleksi Au-oxo6 i Au2phen imaju jake citotoksične efekte. Međutim, te studije su pokazale da je najverovatnije reč o mehanizmu koji je „DNK nezavisan“ i da uključuje inhibiciju tioredoksin reduktaze što dovodi do oštećenja mitohondrija i pokretanja apoptoze. U svakom slučaju, zanimljivo je da i ovi kompleksi imaju bipiridinske ligande što zajedno sa ovde prikazanim rezultatima počinje da formira sistem koji upućuje na to da je bipiridinski strukturni motiv nosilac biološke aktivnosti [8, 65, 116].

Uočena je korelacija između stepena inhibicije Na^+/K^+ ATPaze i vrednosti CBPI. Porast inhibicije doveo je do opadanja indeksa proliferacije što je u saglasnosti sa sličnim rezultatima dobijenim na kardijačne glikozide biljaka [117]. Ovo je očekivano, jer je već navedeno da je Na^+/K^+ ATPaza uključena u procese ćelijske signalizacije i preživljavanja.

Ovo svojstvo novih jedinjenja zlata povećava njihov značaj kao potencijalnih antitumorskih lekova.

U uslovima simulirane biološke sredine pokazano je da dolazi do podjednako jake inhibicije Na^+/K^+ ATPaze i da su HPLC metoda za merenje aktivnosti Na^+/K^+ ATPaze i standardni enzimski test u dobroj saglasnosti oko tih rezultata. Usled praktičnih ograničenja nisu pravljeni uzorci sa višim koncentracijama inhibitora, ali je evidentno da se inhibicija odvija na istovetan način kao i u ostalim enzimskim testovima, što je u skladu sa očekivanjima. Ovime je pokazano da čak i uslovima koji vladaju unutar organizma ovi kompleksi pokazuju visok stepen inhibicije Na^+/K^+ ATPaze i ne bivaju vezani od strane drugih proteina i malih molekula pre nego što iskažu svoju biohemijsku funkciju. Važno je naglasiti da su ova merenja rađena u uslovima simulirane *in vivo* sredine, u uzorcima humane krvi koja je uzeta od zdravih pacijenata. Pošto su uzorci tretirani kompleksima nakon uzimanja krvi, isključen je uticaj organskih sistema i tkiva na ove komplekse, te se stoga ne može pouzdano tvrditi da bi isti efekti nastupili i unutar živih organizama, premda je to veoma verovatno, na osnovu rezultata ovog rada. Potrebno je dalje ispitivanje u *in vivo* uslovima kako bi se dali odgovori na ova pitanja.

Maseni spektri daju dobar uvid u ponašanje kompleksa zlata u prisustvu donora –SH grupe. U spektru cistein hidrohlorida ne zapaža se ništa neobično, molekulski jon $MH2^+$ je mase 122,90, a prisutan je i cistin, sastavljen od dva molekula cisteina spojena –S–S– vezom, mase 242. Ovaj spektar dobijen je uz upotrebu DHB matriksa (2,5 – dihidroksibenzoatna kiselina) u premiks tehnici (matriks i uzorak su umešani u jednakim zapreminama pre nanošenja). Za snimanje masenih spektara AubipyOH i mešavine AubipyOH i cisteina nije korišćen matriks, jer AubipyOH dobro jonizuje usled prisustva bipiridinskog liganda koji dobro apsorbuje svetlost UV jonizacionog lasera. U spektru kompleksa se vide grupe pikova koje potiču od kompleksa (masa molekulskog jona 375 i masa MH^+ jona 376) ali su prisutni i prateći pikovi. Neki od ovih pratećih pikova su nastali delimičnim raspadanjem kompleksa pod uslovima snimanja, kao na primer pikovi grupisani oko mase 361, nastali gubitkom jednog OH^- liganda ili nastali primanjem molekula vode (pikovi oko mase 393). Problem u dobijanju jasnog spektra pravi činjenica da zlato poseduje nekoliko izotopa koji

utiču na pojavu pratećih pikova, ali je prilično jasno da je prisutan molekulski jon kompleksa AubipyOH. Ovde je važno napomenuti da su tokom snimanja uočeni pikovi sa masama od 197 i njihovi umnošci, od kojih je jedan vidljiv i na ovom spektru, na masi 395. Ovi pikovi potiču od atomskog zlata sa same pločice za nanošenje analita. Koincidentalno, ovaj pik upada u opseg blizu mase kompleksa i važno ga je isključiti iz razmatranja spektra. On ostaje jasno vidljiv u spektru smeše cisteina i kompleksa. U masenom spektru uzorka smeše cisteina i kompleksa AubipyOH vrlo jasno se vidi da kompleks biva u potpunosti razoren i da preostaje samo pik na masi 318, koji odgovara kompleksu Au-Cys. Prisutan je i pomenuti jon 395 koji potiče od pločice za uzorke. Ostaje nerazjašnjeno zašto ne dolazi do formiranja molekulskih vrsta u kojima je prisutno više molekula cisteina. Moguće je da pod uslovima koji vladaju u uzorku ne nastaju viši kompleksi cisteina i zlata, ili da dolazi do redukcije zlata do Au (I) ili Au^0 oblika, pa da nastaje organometalno jedinjenje Au-S-CH_2-CH(NH_3^+)-COOH. U prilog organometalnom jedinjenju ide i činjenica da je masa od 318 za jedan manja od mase zbira zlata (197) i cistein hidrohlorida (122). Ipak, bez detaljnije analize nije moguće reći kakva je tačna struktura jona mase 318. U toku ove serije merenja pripremani su i snimani uzorci koji su sadržavali čist komercijalni enzim Na^+/K^+ ATPaze iz svinjskog mozga, kao i smešu enzima i kompleksa AubipyOH. Međutim, spektar u kojem se vidi signal enzima nije dobijen. Razlog za to je što kompleks enzima ima izuzetno veliku masu i ne može se snimati sa trenutnim mogućnostima instrumenta.

Iz prikazanih rezultata se uočava značaj ispitivanih jedinjenja kao potencijalnih antitumorskih terapeutika, kao što je ranije pretpostavljeno u studijama [6, 8, 53, 56, 61, 65, 102]. Kompleksi koji sadrže piridinske derivate kao ligande pokazali su visoku citotoksičnost u uslovima koji nalikuju onima koji postoje u humanom organizmu, što je u saglasnosti sa ranijim studijama analoga na bazi cinka i rutenijuma [114, 115]. Postoje rizici od neželjenog toksičnog delovanja ovih jedinjenja, jer su ovi kompleksi pokazali visoku sposobnost inhibicije Na^+/K^+ ATPaze.

Kada je uočen efekat povećanja enzimske aktivnosti na preparatu sinaptozomalnih plazma membrana od strane koloida C3, jedna od prvih hipoteza bila je da dolazi do imobilizacije enzima na površini nanočestica i do povećanja aktivne površine SPM. Ovo se kasnije

pokazalo kao tačno. Međutim, nije bilo jasno koja interakcija na molekulskom nivou diktira tu imobilizaciju. –SH grupe enzima bi svakako mogle biti odgovorne za tu interakciju ali kao što je gore pokazano takvo vezivanje dovodi do gubitka enzimske aktivnosti Na^+/K^+ ATPaze. Prva stvar koja je proverena nakon što je ovaj efekat uočen bila je interakcija između prečišćenog, komercijalno dostupnog enzima i nanočestica zlata. Efekat povećanja aktivnosti je u tom slučaju izostao, što navodi na to da je za interakciju sa nanočesticom zlata odgovorna membrana ćelije, koja je prisutna u preparatu SPM. Pri višim koncentracijama koloida uočena je blaga inhibicija, koja potiče od zaostalih slobodnih jona zlata koji nisu redukovani tokom sinteze. Ova inhibicija se takođe može sprečiti dodatkom nekog –SH donora, no ovi rezultati nisu prikazani jer nisu od značaja, već su kuriozitet. Dodatak –SH donora u slučaju C3 nije poremetio porast aktivnosti, što dodatno ukazuje na to da se interakcija nanočestica i SPM ne odvija preko –SH grupa. Kao i u eksperimentima sa kompleksima zlata i ovde je kontrolno praćena aktivnost Na^+/K^+ nezavisnih ATPaza. Kod njih nije uočen efekat povećanja aktivnosti, što je inicijalno i bio prvi nagoveštaj da je verovatno reč o nekom efektu gde bi membrana sama bila posrednik.

U testu sa uabainom kao merilom selektivnosti pokazano je da se metoda imobilizacije na površini nanošestice zlata može iskoristiti za razvijanje novih, osetljivijih testova za merenje aktivnosti Na^+/K^+ ATPaze u membranama ćelije. Štaviše, efekat povećanja aktivne površine ćelijske membrane znači da bi povećanje relativne aktivnosti enzima bilo uočeno i kod drugih enzima koji su pozicionirani u membrani, jer po vezivanju za koloid C3 relativno veći broj molekula enzima koji je ugrađen u membranu postaje dostupan za reakciju sa supstratom.

Promena intenziteta plazmonskog pika sa vremenom koja je uočena u spektrofotometrijskim eksperimentima sa koloidom i preparatom SPM govori o brzini te reakcije. Interesantno je i zapažanje promena organoleptičkih svojstava i teksture rastvora nanočestica do kojih dolazi stajanjem rastvora duže od 1 h na sobnoj temperaturi bez mešanja i mućkanja. Naime, vremenom postaju vidljivi vrpčasti agregati vrlo fine i nestabilne strukture, nalik na paučinu, tamno crvene do potpuno crne boje. Ova pojava

izostaje kod čistog koloida ili je potrebno nekoliko nedelja mirovanja da bi se uočio bilo kakav talog na dnu suda.

U spektru koloida C2 ne vide se gotovo nikakve promene, što upućuje na to da ne postoji nikakva interakcija između koloida i SPM. Ni nakon dužeg stajanja (~24h) ne uočava se nikakav talog, niti promena organoleptičkih svojstava rastvora. Jedina promena koja je vidljiva na spektru jeste malo pomeranje plazmonskog pika sa 525 nm na približno 530 nm, koje može upućivati na to da je došlo do neke vrste adsorpcije SMP na površini koloida. Plazmonski pik menja svoju poziciju u spektru u zavisnosti od veličine čestica, pa je moguće da prisustvo SPM dovodi do grupisanja nanočestica u veće klastere [118]. Kod koloida C1 je formiranje taloga veoma brzo i prati ga vidljivo obezbojenje rastvora i formiranje stabilnog precipitata. Međutim, kako koncentracije u spektrofotometrijskim snimanjima ne odgovaraju onima u enzimskom testu, teško je praviti paralele između događanja u testu i ovde.

Kada je reč o FTIR merenjima, tu postoji nekoliko problema, koji uglavnom proizilaze iz visoke hemijske složenosti (heterogenosti) materijala kao što je ćelijska membrana. Tumačenje dobijenih rezultata je nezahvalno i ne daje odlučujuće odgovore na pitanja vezana za ovu problematiku. Spektri, iako informativni (dozvoljavaju uvid u dinamiku kojom se reakcija između koloida i SPM odigrava) ne daju konkretne odgovore o molekulskim grupama uključenim u ove interakcije. Neki zaključci ipak mogu da se izvedu. Promene u regionu oko 1500 – 1600 cm^{-1} i koje su prisutne kod koloida C1 i C2, a izostaju kod koloida C3 potiču od citratnih jona koji su prisutni u materijalu, jer su C1 i C2 citratom stabilisane nanočestice, dok je C3 boratom stabilisana nanočestica. Promene koje se javljaju na 1650 i 1537 cm^{-1} pokazuju da u omotaču koloidne čestice dolazi do izmena molekula citrata, najverovatnije za neke od molekulskih vrsta koje su prisutne u membrani (fosfolipidi, amini, proteini, saharidi). Ova činjenica, uz podatak da ova dva koloida nisu dovela do pojačanja aktivnosti enzima u preparatu jasno ukazuje na to da hemijska interakcija ne implicira efekat povećanja enzimske aktivnosti, tj. da postoji više paralelnih fizičko hemijskih procesa koji se odvijaju, od kojih samo neki dovode do "razlivanja" membranskih fragmenata po površini nanočestice i povećanja aktivnosti enzima. Kada je

reč o spektrima koloida C3 oni su još teži za tumačenje jer je boratni jon teško vidljiv u ovakvim reakcionim uslovima. Ono što se na spektrima uočava je i promena u regionu oko 2900 cm^{-1}, koja je posledica asimetričnog rastezanja metilenskih grupa i lipidnom omotaču i pojačanje njihovog signala koje je posledica imoblilizacije na površini nanočestice. Efekat površinski pojačanog Ramanovog rasipanja je vrlo izražen i doprinosi promeni intenziteta ovih pikova. Slično se događa i sa proteinskim trakama na oko 1060 cm^{-1} koje takođe menjaju svoj intenzitet u svim prikazanim spektrima, a odnose se na rastezanja C-O i C-N veza. Na većim talasnim dužinama oko 800 do 900 cm^{-1} mogu se uočiti simetrična rastezanja νAu-S veze, ali se ona jedva razlikuju od šuma i njihovo tumačenje nije pouzdano.

Iz dobijenih rezultata dobija se nekoliko važnih podataka. Pre svega, deo interakcije između koloida i SPM je trenutan ili veoma brz, kao što se pokazalo u ispitivanjima aktivnosti enzima nakon izlaganja enzima koloidu. S druge strane prisutna je i jedna spora, ali konstantna reakcija koja se razvija tokom više od 20 minuta i koja se može pratiti na FTIR spektrometru. Ova reakcija nema uticaja na enzim koji je prisutan u membrani i najverovatnije ga ne uključuje. Dolazi do izmena u strukturi omotača nanočestice i to je ono što najverovatnije tokom vremena i dovodi do formiranja precipitata i kolapsa suspenzije.

Vizualizacija događaja do kojih dolazi po izlaganju SPM koloidima pomoću AFM metode izuzetno dobro prikazuje fizičke promene u materijalu do kojih dolazi. Koloidi C1 i C3 pokazali su veliki afinitiet ka slepljivanju sa SPM materijalom, s tom razlikom da slepljivanje sa krupnijim česticama koloida C1 dovodi do formiranja agregata koji nemaju bitno izmenjenu aktivnu površinu, dok slepljivanje sa sitnim česticama koloida C3 dovodi do cepanja krupnih “grudvi” SPM na sitne “pačeve” ili “kapljice” koje ostvaruju bolji kontakt sa vodenom sredinom u kojoj se nalazi supstrat enzima i time dovode do prividno povećane aktivnosti, jer je veći broj molekula enzima dostupan za reakciju. Razlog za ovo je velika pojedinačna kontaktna površina između nanočestica koloida C1, koja omogućuje da nanočestice ostanu slepljene pomoću SPM. Sile koje na tom nivou veličine vladaju, pre svega površinski napon ćelijske membrane koji diktira njeno ponašanje, ne dovode do

cepanja krupnih agregata na sitnije. Sasvim je moguće da bi neki mehanički tretman, na primer ultrazvuk ili intenzivno mešanje mogao da dovede do usitnjavanja SPM na isti način, ali proteini kao nestabilni molekuli, a naročito Na^+/K^+ ATPaza, loše podnose takve agresivne mehaničke tretmane i lako se raspadaju u tim uslovima.

Uopšteno, svi dobijeni rezultati za komplekse i koloide zlata u dobroj su saglasnosti sa do sada poznatim i relevantnim rezultatima iz literature u onim slučajevima gde slični rezultati postoje.

6. Zaključak

6.1. Kompleksi zlata

Na osnovu prikazanih rezultata i njihove diskusije može se sa sigurnošću zaključiti da kompleksi $[AuCl_4]^-$, AuDMSO, Aubipy, AubipyOH, Aupy i AubipyC vrše dozno zavisnu inhibiciju ATPazne aktivnosti enzima Na^+/K^+ ATPaze izolovane iz mozga pacova, mozga svinje i membrana humanih ćelija krvi. Srednje inhibicione koncentracije su izmerene za sve komplekse koji su pokazali inhibiciju i kreću se u opsegu od 10^{-7} do 10^{-6} M u zavisnosti od kompleksa. Inhibicione konstante za prečišćen komercijalni enzim (iz mozga svinje) su izmerene i kreću se u opsegu od 10^{-6} do 10^{-5} M u zavisnosti od kompleksa. Stoga se zaključuje da je reč o umereno jakim inhibitorima koji su prema potenciji uporedivi sa kardiotoničnim glikozidima koji su u kliničkoj upotrebi. Na osnovu urađene kinetičke studije na prečišćenom komercijalnom enzimu iz mozga svinje, zaključuje se da su prema tipu inhibicije ovi kompleksi nekompetitivni inhibitori Na^+/K^+ ATPaze iz mozga svinje. Osim toga, kao što je pokazano u eksperimentima sa donorima –SH grupe, tačnije cistein hidrohloridom i redukovanim glutationom, ova inhibicija je reverzibilna i može se gotovo potpuno otkloniti dodatkom cisteina ili glutationa. Štaviše, ukoliko su pre dodavanja kompleksa zlata u rastvoru prisutne jednake ili veće koncentracije cisteina ili glutationa, ovu inhibiciju je moguće u potpunosti predupreditи. Na osnovu ovoga bezbedno je zaključiti da se vezivanje ovih kompleksa za enzim odvija posredstvom slobodnih –SH grupa koje su brojne i prisutne na površini enzima, ali da nije sigurno koliki broj ovih grupa je uključen u vezivanje, kao što nisu sigurne ni lokacije vezujućih cisteinskih ostataka u primarnoj strukturi. Ispitivanja na kulturama humanih limfocita pokazala su da su kompleksi koji sadrže bipiridinske ligande (kompleksi Aubipy, AubipyOH, Aupy i AubipyC) izuzetno citotoksični i da dovode do blokiranja proliferacije ćelija i pojave mikronukleusa, a pri višim koncentracijama dovode do nekroze ćelija. Uočava se jasna korelacija između structure liganda i biološkog efekta – kompleksi koji sadrže bipiridinske

ili slične aromatične ligande pokazuju visoku citotoksičnost. Kompleksi $[AuCl_4]^-$ i AuDMSO nisu pokazali značajnu citotoksičnost. Aktivnost kompleksa Au-oxo6, Au_2phen i Aucyclam na kulturama ćelija nije ispitivana jer je poznata iz literature i u saglasnosti je sa rezultatima našeg rada. Na osnovu prikazanih rezultata zaključuje se da mehanizam citotoksičnog delovanja kompleksa Au-oxo6, Au_2phen i Aucyclam ne uključuje inhibiciju ATPazne funkcije Na^+/K^+ ATPaze. Na uzorcima prečišćenih membrana ćelija tretirane humane krvi je pokazano da čak i u uslovima koji vladaju u humanom tkivu kompleksi $[AuCl_4]^-$, AuDMSO, Aubipy, AubipyOH, Aupy i AubipyC zadržavaju svoju sposobnost da inhibiraju Na^+/K^+ ATPazu i da im prisustvo drugih protiena i –SH donora ne umanjuje aktivnost dramatično. Umanjenje aktivnosti inhibitornog potencijala kompleksa je ipak primećeno u manjoj meri.

Tokom ovog eksperimenta upotrebom HPLC metode za detekciju i kvantifikaciju nastalog ADP pokazano je da je ova metoda u dobroj saglasnosti sa tradicionalnim testom za merenje aktivnosti Na^+/K^+ ATPaze preko količine oslobođenog fosfata spektrofotometrijskom metodom.

U eksperimentima na MALDI TOF masenom spektrometru je na primeru kompleksa AubipyOH pokazano da postoji direktna reakcija između cisteina i ovog kompleksa koja dovodi do uništenja kompleksa i formiranja molekulske vrste mase 318 koja može biti kompleks zlata i cisteina. Ovo objašnjava ranije pokazani mehanizam sprečavanja inhibicije Na^+/K^+ ATPaze. Iako merenja iz tehničkih razloga nisu urađena za svaki kompleks ponaosob, može se zaključiti da je ova reakcija prisutna i u slučaju sprečavanja inhibicije izazvane drugim kompleksima koji su pokazali inhibitorno delovanje.

6.2. Koloidi

Nanočestice koloida C1 i C2 ne pokazuju značajan efekat na preparat Na^+/K^+ ATPaze iz sinaptozomalnih plazma membrana (SPM) ćelija mozga pacova. Mala inhibicija koja je

prisutna posledica je zaostalih neredukovanih jona zlata i može se u potpunosti otkloniti tokom rada uz dodatak 10^{-6} M cisteina. Ova inhibicija je artefakt, direktnog delovanja nanočestica na aktivnost Na^+/K^+ ATPaze nema. S druge strane, koloid C3 pokazao je dramatičan efekat na aktivnost Na^+/K^+ ATPaze u preparatu SPM. Koloid C3 je izazvao povećanje aktivnosti ovog enzima za preko 100% , a pri tom nije izazvao povećanje aktivnosti pratećih ATPaza u preparatu. Ovaj porast aktivnosti ne zavisi od vremena preinkubacije i stabilan je tokom 60 minuta, nakon čega počinje polako da opada tokom narednih sati, kada se i formira vidljiv talog na dnu suda. Selektivnost enzima tretiranog nanočesticama koloida C3 za njegov prirodni ligand i inhibitor uabain ostaje neizmenjena, a signal testa je pojačan. U Vis spektrima koloida C1 i C3 se uočavaju značajne promene po dodatku preparata SPM. Oblik i pozicija plazmonskog pika na 525 nm se menjaju tokom 20 minuta, što dovodi do zaključka da pored efekta na povećanje aktivnosti Na^+/K^+ ATPaze u uzorku postoji i druga, sporija interakcija koja dovodi do kolapsa nanočestice. Kod koloida C2 ovo nije uočeno i bilo kakva interakcija sa preparatom SPM je izostala. Iz FTIR spektara se može zaključiti da je prisutna jedna spora interakcija između SPM i nanočestica koloida C1, C3 i u manjoj meri C2, ali da ta interakcija nije uzrok povećanja aktivnosti Na^+/K^+ ATPaze. Na osnovu promena u karakterističnim pikovima zaključuje se da dolazi do hemijske izmene u površini koloidne sfere nanočestice sa fosfolipidnim slojem u membranama preparata SPM. Ova interakcija nije dublje istraživana. Konačna potvrda hipoteze da povećanje aktivnosti Na^+/K^+ ATPaze nije posledica biohemijskog delovanja nanočestice na sam enzim, već fizičko-hemijskog delovanja na preparat SPM u celosti dolazi sa AFM mikrografija, gde se jasno uočava da se u prisustvu nanočestica koloida C3 gubi vrpčasta i zgrudvana struktura koju SPM zauzima nakon odvajanja ultracentrifugiranjem i gde nastaju fino dispergovani fragmenti membrana. Ovako dispergovani fragmenti SPM imaju povećanu kontaktnu površinu i veći broj molekula enzima je dostupan za reakciju sa supstratom, što dovodi do prividnog povećanja aktivnosti enzima. U smeši koloida C1 i SPM dolazi do promena u strukturi koje su vidljive na AFM i FTIR, ali izostaje efekat na aktivnost enzima. Koloid C2 u korišćenim uslovima ne daje jasno uočljivu promenu na AFM.

Iz svega ovde prikazanog zaključuje se da kompleksna jedinjenja zlata svakako imaju potencijal za upotrebu u medicini, jer pokazuju visok stepen reaktivnosti sa važnim molekulskim metama. Koloidne nanočestice zlata pokazuju atraktivna fizička svojstva na nano nivou i predstavljaju potencijalno sredstvo za nano manipulaciju materijala biološkog porekla, koje može biti od značaja za industriju i nauku.

7. Eksperimentalni deo

7.1. Preparati enzima

7.1.1. Komercijalni enzim

Komercijalni preparat enzima (Sigma, A7510-5UN) i prema deklaraciji predstavlja enzim izolovan iz korteksa svinje (*Sus scrofa)* i sadrži ~10% proteina, 90% saharoze, 0.4% EDTA (etilen - diamin – tetraacetatna kiselina) i 0.06% NaCl. Preparat je čuvan u čvrstom stanju do upotrebe, jer je primećeno da rastvor čuvanjem na -20 ºC gubi aktivnost. Radni rastvor enzima imao je koncentraciju 5mg čvrstog preparata po mililitru 50 mM TrisHCl pufera pH=7,4 (Sigma). Ovako pripremljen enzim korišćen je za "skrining" inhibitorne aktivnosti kompleksa i za ispitivanje kinetike inhibicije. U daljem tekstu ovaj preparat enzima biće navođen kao čist enzim.

7.1.2. Izolovanje sinaptozomalnih plazma membrana iz mozga pacova

Pored čistog enzima, u radu sa koloidnim nanočesticama je korišćen preparat sinaptozomalnih plazma membrana (SPM) izolovanih iz mozga pacova (*Rattus norvegicus*) linije Vistar (Wistar). Žrtvovanje svih životinja odobreno je od strane etičkog komiteta Medicinskog fakulteta Univerziteta u Beogradu. Postupak za izolovanje SPM je sledeći. Nakon žrtvovanja mozak životinje se izoluje i ispira u 0,32 M saharozi (Zorka) puferisanoj na pH=7,4 sa 10 mM TrisHCl. Zatim se mozak homogenizuje u 10 mL hladne puferisane saharoze. Svi dalji koraci izvode se na hladnom, idealno oko 4 ºC. Homogenat se centrifugira na 1000 × g tokom 10 minuta da bi se istaložila jedra, a sakuplja se supernatant. Sinaptozomi se iz supernatanta talože centrifugiranjem na 10000 × g tokom 20 minuta. Talog se zatim resuspentuje u 3 mL puferisane saharoze i nanosi se na diskontinualni gradijent puferisane saharoze i to: 1,2 M 12 mL, 1 M 6 mL i 0,8 M 6 mL. Nakon centrifugiranja na 90000 × g tokom 105 minuta sakuplja se frakcija koja ostaje na prelazu između sloja od 1,2 M i 1 M saharoze, razblažuje se puferisanom 0,25 M saharozom i taloži na 15000 × g tokom 20 minuta i taj talog predstavlja prečišćene

sinaptozome. Oni se liziraju zamrzavanjem preko noći, a potom se dodatno prečišćavaju ponavljanjem navedenog koraka sa ultracentrifugiranjem u gradijentu saharoze. Na taj način se dobijaju sinaptozomalne plazma membrane koje se razblažuju do koncentracije od 1mg proteina po mililitru i čuvaju se u 10 mM TrisHCl puferu pH=7,4.

7.1.3. Izolovanje membrana ćelija humane krvi

U eksperimentima u kojima se merila aktivnost Na^+/K^+ ATPaze u membranama humanih ćelija korišćeni su tzv. "duhovi", tj. membrane ćelija krvi, pretežno eritrocita, iz pune ljudske krvi koja je prethodno bila tretirana željenom koncentracijom kompleksa zlata. Membrane su dobijene tako što su ćelje lizirane u hipotoničnom rastvoru, a membrane odvojene centrifugiranjem i isprane. Suspenzije membrana takođe su razblažene do 1mg proteina po mililitru i čuvane su u 10 mM TrisHCl puferu pH=7,4. Krv je sakupljana od zdravih, mladih, muških dobrovoljaca, nepušača, u skladu sa zdravstvenom i etičkom regulativom Republike Srbije [119].

7.2. Enzimski testovi

Aktivnost Na^+/K^+ ATPaze koja je glavni parametar u ovom radu praćena je na dva načina. Prvi i glavni je pomoću testa za određivanje koncentracije fosfata [44], a drugi je preko HPLC metode za određivanje ADP. Obe metode su opisane niže.

7.2.1. Osnovni test

Osnovni test za određivanje aktivnosti Na^+/K^+ ATPaze preko količine nastalog fosfata izvodi se na sledeći način. U epruvetu se sipa 50 μL totalne inkubacione smeše (0,4 M NaCl (Sigma); 0,08 M KCl (Sigma); 0,02 M $MgCl_2$ (Sigma); 0,2 M TrisHCl pH=7,4), zatim 25 μL uzorka enzima, 105 μL dejonizovane vode (pravljene u laboratoriji na Millipore Milli-Q sistemu) i nakon kratke predinkubacije od 5 minuta na 37 ºC u vodenom kupatilu dodaje se 20 μL 20 mM TrisATP (Sigma). Inkubacija traje 10-15 minuta i prekida se pomoću 22 μL ledene 3 M $HClO_4$ (Merck). Potom se epruveta stavlja u ledeno kupatilo do početka postupka za bojenje fosfata.

Ovaj osnovni test može pratiti test za sporedne ATPaze, ukoliko su one prisutne u uzorku enzima (npr. u SPM ili membranama humanih ćelija krvi). Ovaj test se izvodi na istovetan način, sa tom razlikom što se umesto 50 μL totalne inkubacione smeše koristi 50 μL magnezijumske inkubacione smeše koja ne sadrži jone natrijuma i kalijuma (0,02 M $MgCl_2$; 0,2 M TrisHCl pH=7,4). Aktivnost dobijena u ovom testu se oduzima od one dobijene u testu sa totalnom inkubacionom smešom.

U radu su korišćene i varijante ovog testa u koje su dodavani rastvori kompleksa, koloida, uabaina (Sigma), cistein hidrohlorida (Sigma) ili redukovanog glutationa (Sigma), na račun navedenih 105 μL vode.

7.2.2. Inhibicioni test

Kada se meri inhibicija ili aktivacija enzima, u epruvetu se sipa 50 μL totalne inkubacione smeše (0,4 M NaCl; 0,08 M KCl; 0,02 M $MgCl_2$; 0,2 M TrisHCl pH=7,4), zatim 25 μL uzorka enzima, 85 μL dejonizovane vode i 20 μL ispitivanog inhibitora u željenoj koncentraciji i nakon predinkubacije od 10 minuta na 37 ºC u vodenom kupatilu dodaje se 20 μL 20 mM TrisATP. Inkubacija traje 10-15 minuta i prekida se pomoću 22 μL ledene 3 M $HClO_4$ (Merck). Potom se epruveta stavlja u ledeno kupatilo do početka postupka za bojenje fosfata.

7.2.3. Test za merenje sprečavanja inhibicije

Za merenje sprečavanja inhibicije postupak je sledeći: U 50 μL totalne inkubacione smeše (0,4 M NaCl; 0,08 M KCl; 0,02 M $MgCl_2$; 0,2 M TrisHCl pH=7,4), doda se 25 μL uzorka enzima, 85 μL dejonizovane vode i 20 μL rastvora cisteina ili glutationa, u zavisnosti od toga koji se –SH donor ispituje. Potom se sipa 20 μL ispitivanog jedinjenja zlata u željenoj koncentraciji i nakon predinkubacije od 10 minuta na 37 ºC u vodenom kupatilu dodaje se 20 μL 20 mM TrisATP. Inkubacija traje 10-15 minuta i prekida se pomoću 22 μL ledene 3 M $HClO_4$ (Merck). Potom se epruveta stavlja u ledeno kupatilo do početka postupka za bojenje fosfata.

7.2.4. Test za merenje reaktivacije inhibiranog enzima

Za merenje reaktivacije inhibiranog enzima postupak je sledeći. U epruvetu se sipa 50 μL totalne inkubacione smeše (0,4 M NaCl; 0,08 M KCl; 0,02 M $MgCl_2$; 0,2 M TrisHCl pH=7,4), zatim 25 μL uzorka enzima, 85 μL dejonizovane vode i 20 μL ispitivanog jedinjenja zlata u željenoj koncentraciji. Nakon 10 minuta inkubacije na 37 ºC dodaje se 20 μL rastvora cisteina ili glutationa, u zavisnosti od toga koji se –SH donor ispituje. Nakon predinkubacije od 10 minuta na u vodenom kupatilu dodaje se 20 μL 20 mM TrisATP. Inkubacija zatim traje 10-15 minuta i prekida se pomoću 22 μL ledene 3 M $HClO_4$ (Merck). Potom se epruveta stavlja u ledeno kupatilo do početka postupka za bojenje fosfata.

7.2.5. Test za kinetička merenja

Test za kinetička merenja se razlikuje u odnosu na osnovni test u pogledu koncentracija i vremena inkubacije. Naime, u epruvetu se sipa 50 μL totalne inkubacione smeše (0,4 M NaCl 0,08 M KCl; 0,02 M $MgCl_2$; 0,2 M TrisHCl pH=7,4), zatim 25 μL uzorka čistog komercijalnog enzima, 105 μL dejonizovane vode i nakon kratke predinkubacije od 5 minuta na 37 ºC u vodenom kupatilu dodaje se TrisATP u željenoj koncentraciji (između 1 i 5 mM finalno). Inkubacija traje tačno 240 sekundi i prekida se pomoću 22 μL ledene 3 M $HClO_4$ (Merck). Potom se epruveta stavlja u ledeno kupatilo do početka postupka za bojenje fosfata.

7.2.6. Test za kinetička merenja sa inhibitorom

Kada se određuju kinetički parameti za inhibiran enzim postupak je sličan. U epruvetu se sipa 50 μL totalne inkubacione smeše (0,4 M NaCl 0,08 M KCl; 0,02 M $MgCl_2$; 0,2 M TrisHCl pH=7,4), zatim 25 μL uzorka čistog komercijalnog enzima, 85 μL dejonizovane vode i 20 μL kompleksa enzima u koncentraciji 10 puta većoj od one koja daje IC_{50} (tako da je finalna koncentracija inhibitora upravo IC_{50}). Nakon kratke predinkubacije od 5 minuta na 37 ºC u vodenom kupatilu dodaje se TrisATP u željenoj koncentraciji (između 1 i 5 mM finalno). Inkubacija traje tačno 240 sekundi i prekida se pomoću 22 μL ledene 3 M $HClO_4$ (Merck). Potom se epruveta stavlja u ledeno kupatilo do početka postupka za bojenje fosfata.

7.2.7. Test za membrane humanih ćelija

Za testove u kojima su korišćene membrane humanih ćelija krvi je potrebno nakon zaustavljanja reakcije izvršiti kratko i jako centrifugiranje (5 minuta na 13400 obrtaja je dovoljno) da bi se oborio talog denaturisanih proteina. Kao uzorak se uzima 200 μL supernatanta.

7.2.8. Bojenje fosfata

Nakon završenog testa, bez obzira na tip testa, bojenje fosfata vršeno je tako što je najpre u zaustavljenu reakcionu smešu dodato 4,5 mL dejonizovane vode i 200 μL 0,02 M amonijum heptamolibdata ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, Merck) u 30% H_2SO_4. Potom je u epruvetu dodata kap (~33 μL) 132 mM $SnCl_2$ (Merck) u anhidrovanom glicerolu (Zorka). Smeša je promućkana na mućkalici (Vortex) i nakon 15 minuta merena je apsorbanca na 690 nm (Perkin Elmer Lambda 35 UV-vis spektrofotometar, 1 cm optički put). Izmerena apsorbanca je po oduzimanju vrednosti slepe probe korišćena kao mera relativne enzimske aktivnosti.

7.2.9. Hromatografska metoda za merenje aktivnosti enzima

U radu je korišćena HPLC metoda za određivanje adeninskih nukleozida, pre svega ADP, koji je glavni proizvod enzimske aktivnosti. Nakon završene enzimske reakcije uzorci su podešavani na pH 7 i filtrirani, a zatim snimani na HPLC sistemu prema sledećoj metodi.

Korišćen je Waters UPLC sistem sa PDA detektorom i Waters reverzno faznom kolonom BEH C18 sa veličinom čestice nosača od 1,7 μm, dimenzija 100 mm × 2,1 mm. Kao mobilna faza korišćena je smeša 2 mM tetrabutil amonijum hidroksida u 4 mM fosfatnom puferu i metanola u zapreminskom odnosu 71:29. Brzina protoka mobilne faze bila je 0,25 mL/min, a temperatura kolone tokom rada je bila 40 ºC. Detekcija je vršena na 254 nm. Nanošeno je 2 μL uzorka koji je pripremljen isto kao i za gore navedeno bojenje fosfata, uz prethodno podešavanje pH do oko 7. Uzorci su nanošeni automatski. Koncentracije su određivane sa standardne krive napravljene pod istim uslovima za poznate koncentracije analita.

7.3. Analiza koloida spektrofotometrijom

Pored snimanja apsorbanci uzoraka u enzimskom testu, UV-vis spektrometrija je korišćena za snimanje promena u spektrima koloida zlata tokom rada i pod različitim uslovima sredine. Temperatura u svim merenjima bila je oko 20 ºC.

Za potrebe praćenja promena na koloidnim česticama u prisustvo enzima, posmatrana je promena apsorpcionog spektra u regionu od 400 nm do 700 nm. Neposredno pre merenja u 1 mL rastvora SPM koncentracije 100 µg/mL dodaje se 0,5 mL ispitivanog koloidnog rastvora nanočestica zlata koncentracije 10^{-10} M. Spektri su snimani tokom 20 minuta. Pri istim uslovima snimani su nezavisno i spektri koloida pri koncentraciji nanočestica zlata od $3{,}3 \times 10^{-11}$ M , kao i spektri SPM pri koncentraciji od 66,6 µg/mL kao kontrole. Dobijeni spektri obrađeni su u softverskom paketu Origin 8.5.

7.4. Analiza koloida infracrvenom spektroskopijom

FTIR spektroskopija je korišćena za praćenje površinske interakcije između SPM i koloidnih nanočestica zlata. Merenja su rađena na aparatu Thermo Electron Corporation Nicolet 380 FTIR spektrofotometru sa ATR (Attenuated Total Reflection) dodatkom sa dijamantskim vrhom. Uzorci su pripremani neposredno pred snimanje. Najpre su snimljeni čisti uzorci koloida pri koncentraciji od 5×10^{-10} M i čisti uzorci SPM pri koncentraciji od 0,5 mg/mL tokom 30 minuta. Zatim su snimane smeše od 0,5 mL rastvora koloida koncentracije 10^{-9} M i 0,5 mL suspenzije SMP koncentracije 1 mg/mL tokom 30 minuta. Snimanje je vršeno u opsegu od 4000 cm^{-1} do 500 cm^{-1}. Dobijeni spektri obrađeni su u softverskom paketu Origin 8.5

7.5. Analiza koloida atomskom mikroskopijom

AFM snimanja su urađena na komercijalnom AFM VECCO Quadrirexed Multi Mode IIIE sistemu koji je radio u tapkajućem modu na visokoorijentisanom pirolitičkom grafitu (HOPG). Površinska topografija i fazne slike su snimljeni pomoću NanoScience-Team Nanotec GmbH Solid Nitride Cone igle za AFM sa radijusom vrha manjim od 10 nm. Snimanja su ponavljana na površinama između 250×250 nm i 2×2 μm na sobnoj temperaturi. Uzorci su nanošeni na sveže očišćenu površinu HOPG 1×1 cm. Za nanošenje uzoraka korišćen je metod uparavanja kapi. Nanošeni rastvori su bili koncentracije 2×10^{-9} M koloida i 1mg/mL SPM. Za mešane uzorke prethodno je pomešano 100 μL rastvora koloida sa 25 μL suspenzije SPM i ostavljeno da stoji 15 minuta na sobnoj temperaturi pre nanošenja. Svi uzorci su sušeni pre snimanja. Digitalna analiza i obrada snimaka vršena je uz upotrebu softvera WSxM 3.1 [120].

7.6. Analiza kompleksa zlata masenom spektroskopijom

MALDI-TOF MS (Matrix assisted laser desorption ionization – time of flight mass spectroscopy) je korišćena da bi se ustanovila izmena liganada u ispitivanim kompleksima. Merenja su rađena na MALDI-TOF MS instrumentu Voyager Biospectrometry DE Pro Workstation (PerSeptive Biosystems), kalibrisanom prema odgovarajućem matriksu. Uređaj je opremljen pulsnim azotnim laserom od 337 nm. Pritisak komore je održavan oko 3×10^{-5} Pa, dok je napon jonskog izvora bio 20 kV. Sva snimanja su vršena na zlatnom nosaču u pozitivnom linearnom modu. Kumulativni spektri su dobijeni ispaljivanjem oko 300 hitaca iz lasera. Vreme odložene ekstrakcije je bilo 100 ms. Odnos signal – šum je podešen pomoću fabričkog softvera Data Explorer 2.2 a za obradu spektara korišćena je verzija 4.9. Uzorci su bili osušeni rastvori kompleksa AubipyOH u koncentraciji od 10^{-4} M sa ili bez dodatka 10^{-3} M cistein hidrohlorida, kao i čist osušeni cistein hidrohlorid.

Pravljeni su uzorci bez matriksa i sa matriksom. Korišćeni su različiti matriksi u premiks tehnici, kao što je navedeno u rezultatima.

7.7. Ćelijske metode

Parametri citotoksičnosti, incidenca mikronukleusa (MN) i citohalazin B proliferacioni indeks (CBPI) mereni su za komplekse zlata na kulturama humanih limfocita izolovanih iz krvi tri mlada muška pacijenta nepušača, a u skladu sa Etičkom regulativom Republike Srbije. Alikvoti heparinizirane krvi od 0,5 mL dodati su u kulturu sa medijumom za kariotipizaciju PB-max (Invitrogen-Gibco) i tretirani su sa rastućim koncentracijama komplksa zlata od 10^{-8} do 10^{-4} M. Netretirane ćelije služile su kao kontrola.

7.7.1. Merenje broja mikronukelusa

Za ispitivanje incidence mikronulekusa korišćen je ranije opisan metod [121]. Nakon 44 sati inkubacije citokineza je zaustavljena citohalazinom B pri finalnoj koncentraciji od 4μg/ml. Potom su ćelije inkubirane još 28 sati, a zatim su prikupljene i lizirane u hipotoničnom rastvoru (0,28%KCl i 0,45% NaCl) na 37 ºC. Suspenzija je potom fiksirana u metanolu i acetatnoj kiselini (3:1) i nanošena na čista mikroskopska stakla. Nakon sušenja materijal je bojen 2% alkalnom Giemsa bojom. Pod mikroskopom AxioImager A1 (Zeiss) sa 400× ili 1000× puta uvećanjem je brojano minimalno 1000 binuklearnih ćelija i beležen je broj mikronukleusa.

7.7.2. Merenje proliferacionog indeksa

CBPI je računat prema ranije opisanoj metodi [122], prema formuli CBPI = MI + 2MII + 3(MIII + MIV)/N, gde su MI, MII, MIII i MIV bojevi ćelija sa po 1,2,3 ili 4 nukleusa, respektivno, a N je broj prebrojanih ćelija. Statistička analiza rezultata rađena je softverima Statistica 8.0 i Origin 8.0.

8. Literatura

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In vitro effects of some gold complexes on Na^+/K^+ ATPase activity and cell proliferation

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ABSTRACT

The *in vitro* influence of gold(III) complexes, $H[AuCl_4]$, $[Au(DMSO)_2Cl_2]Cl$ and $[Au(bipy)Cl_2]Cl$ (bipy = 2,2′-bipyridine), upon commercially available Na^+/K^+ ATPase activity, purified from porcine brain cortex, was investigated. Additionally, the complexes were tested on human lymphocytes, and incidence of micronuclei and cell proliferation index was determined. Concentration-dependent inhibition of the enzyme for all three compounds was obtained, but with differing potencies. Calculated IC_{50} from Hill analysis were (in M): 5.75×10^{-7}, 5.50×10^{-6} and 3.98×10^{-5}, for $H[AuCl_4]$, $[Au(DMSO)_2Cl_2]Cl$ and $[Au(bipy)Cl_2]Cl$, respectively, while Hill coefficient values, n, were above 1 in all cases. This inhibition can be prevented using –SH donating ligands such as L-Cys and glutathione, and these ligands can also cause a recovery of the enzyme activity after the induced inhibition. Kinetic analysis demonstrated that each of the studied gold(III) complexes affects Na^+/K^+ ATPase reducing maximum enzymatic velocity, V_{max}, but not significantly changing the affinity for the substrate (K_M value), implying a noncompetitive mode of the interaction. Furthermore, among investigated gold(III) complexes, the $[Au(bipy)Cl_2]Cl$ complex exhibits a strong cytotoxic effect on human lymphocytes, which suggests its potential for use in antitumor therapy.

1. Introduction

The interest in the chemistry of gold (Au) compounds has received much attention over the last two decades because of their traditional use in medicine for the treatment of various diseases [1,2]. Au(III) complexes are isoelectronic and isostructural with Pt(II) compounds, both metals have the same electronic configuration (d^8) and form square planar complexes. Moreover, Au(III) compounds generally exhibit interesting cytotoxic and antitumor properties and are in the focus of interest in the area of metal based antitumor agents, because of their potential application as alternative to platinum drugs [3–6]. However, Au(III) compounds were neglected for a long time because of their relative instability [6]. It was recently found that some simple mononuclear Au(III) complexes are sufficiently stable under the physiological environment and display relevant cell killing properties toward selected human tumor cell lines [7–9]. Since Pt(II) compounds express antitumor properties most probably due to the interaction with DNA [10], recent investigations pointed out that the interactions of cytotoxic Au(III) complexes with DNA are significantly different and weaker than those of platinum analogues [7,11]. Some gold compounds that were evaluated displayed biological effects that are mediated by an antimitochondrial mechanism rather than by direct DNA damage [8].

Substitution reactions of Au(III) complexes are faster than the d^8 Pt(II) square-planar complexes. Au(III) compounds easily reduce to Au(I) in physiological conditions due to their rapid kinetics and higher charge and redox potential [6,12,13]. It was demonstrated that sulfur-containing biomolecules have high affinity for platinum and gold complexes. While the reactions of some amino acids (L-Cys, L-methionine) and glutathione (GSH) with the Pt(II) and Pd(II) are a simple substitution process [14,15], the reaction of L-Cys and L-methionine with Au(III) complex was found to be initially fast, followed by the much slower process, which was attributed to reduction to Au(I) [16].

Na^+/K^+ ATPase (sodium potassium adenosinetriphosphatase, EC. 3.6.3.9) is membrane located enzyme found in all animal cells [17]. Its main function is to catalyze ATP hydrolysis. The energy liberated in this process is used as the driving force for active transport of monovalent cations (Na^+, K^+) through membranes [18]. The activity of this enzyme is very sensitive to the influence of various bioregulators, such as cardiac steroids, transition and heavy metals, as well as metal complexes [19–27]. Various metal ions binding to enzyme sulfhydryl groups have often been implicated in the inhibition of Na^+/K^+ ATPase activity [22,23,28]. Furthermore, nephrotoxicity, ototoxicity *etc.* of platinum anticancer drugs, such as cisplatin and chloroplatinic acid, are related to inhibition of Na^+/K^+ ATPase activity [24,29]. However, gold

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is one of the most potent nonspecific inhibitors of Na^+/K^+ ATPase, with characteristics differing from other metallic inhibitors of this enzyme system [30]. Besides, reduced metallic gold particles may cause cytotoxic effects, and also enhance Na^+/K^+ ATPase activity [31,32].

Recently, our work was concentrated on the *in vitro* investigations of the influence of some Pt(II) group complexes on Na^+/K^+ ATPase activity and the reactivation of the inhibited enzyme in the presence of GSH and L-Cys. Moreover, it was reported that GSH and L-Cys are potent enzyme activity reactivators, since they recover the metal ions that induced inhibition of some enzymes [14,33,34]. The present paper represents the continuation of our work concerning the influence of noble metal complexes on Na^+/K^+ ATPase activity. The interference of $[Au(DMSO)_2Cl_2]^+$ and $[Au(bipy)Cl_2]^+$ (bipy = 2,2′-bipyridine) complexes with Na^+/K^+ ATPase activity in the presence of L-Cys and GSH was studied and compared to the effect of $[AuCl_4^-]$ (Fig. 1). Besides, the study is broadened by the *in vitro* toxicity investigation of these complexes on human lymphocytes as a model system.

2. Experimental

2.1. Chemicals

All commercially available chemicals were of analytical grade. L-Cys (99.5%), GSH and $H[AuCl_4]$ were obtained from Fluka (Switzerland). $[Au(DMSO)_2Cl_2]Cl$ and $[Au(bipy)Cl_2]Cl$, were synthesized according to the published procedures [8,35,36]. The chemical analysis and UV–VIS spectral data were in good agreement with those obtained for the previous preparation. 1×10^{-3} M stock solutions of complexes in DMSO and 1×10^{-2} M ligands were prepared shortly before use. Working solutions were prepared by diluting the stock solutions to desired concentrations. Na^+/K^+ ATPase from porcine brain cortex (specific activity of 0.1 IU/mg solid) as well as some chemicals for medium assay (ATP, NaCl, KCl, $MgCl_2$, and Tris–HCl) were purchased from Sigma-Aldrich (Germany). Chemicals for determination of Na^+/K^+ ATPase activity (stannous chloride and ammonium molybdate) were from Merck (Germany). Deionized water was used throughout. PB-max karyotyping medium for cell culturing was obtained from Invitrogen-Gibco, Paisley, UK. For micronucleus assay, cytochalasin B and Giemsa stain were purchased from Sigma-Aldrich (Germany). Methanol and acetic acid were obtained from VWR (Germany).

2.2. Na^+/K^+ ATPase activity assay

Na^+/K^+ ATPase activity was determined in a standard incubation medium (200 μl), containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 2 mM ATP and protein (2 mg solid/ml) in the presence or absence (control) of the desired concentration of complex. Incubation mixtures were preincubated for 10 min at 37 °C in the presence or absence of inhibitor or water (control). The reaction was started by the addition of ATP, allowed to proceed for 15 min, and interrupted by the addition of the ice cold $HClO_4$ and immediate cooling on ice. The inorganic orthophosphate (Pi) liberated from the hydrolysis of ATP was measured using modified spectrophotometric procedure [37] based on the stannous chloride method, by reading the absorbance at 690 nm. The results are expressed as the mean percentage of enzyme activity relative to the corresponding control value. All experiments were performed in triplicate.

The effect of L-Cys and GSH on the prevention of the Au(III) induced inhibition was measured under the same conditions as described above, with the ligand added to the assay medium before the exposure to metal complex. The reactivating effect was measured by adding L-Cys and GSH after a 15 minute preincubation with the Au(III) complexes.

2.3. Lymphocyte cultures

Blood samples were obtained from three healthy, non-smoking young male volunteer donors in accordance with current Health and Ethical regulations in Serbia [38].

Aliquots of heparinized whole blood (0.5 ml) were placed in cultures containing PB-max karyotyping medium (Invitrogen-Gibco, Paisley, UK)

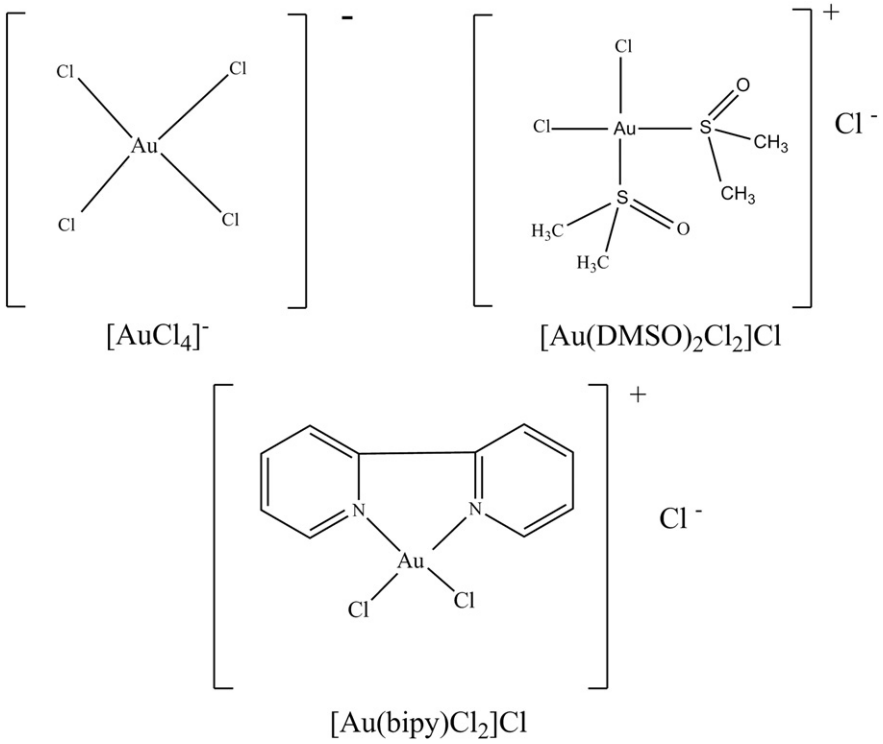

Fig. 1. Structural formulas of the investigated Au(III) complexes.

and treated with increasing concentration of gold complexes (final concentrations from 10^{-8} M to 10^{-4} M). Untreated cell cultures served as controls.

2.4. Micronucleus assay

For micronuclei preparation, the cytokinesis block method of Fenech [39] was used. Cytochalasin B at a final concentration of 4 μg/ml was added to each culture after 44 h of incubation in order to inhibit cytokinesis. The lymphocyte cultures were incubated further for 28 h. Cells were collected by centrifugation and treated with hypotonic solution at 37 °C. Hypotonic solution consisted of 0.56% KCl and 0.90% NaCl (mixed in equal volumes). Cell suspension was fixed in methanol/acetic acid (3:1), washed three times with fixative, and dropped onto a clean slide. Slides were air dried and stained in alkaline Giemsa (2%). For each sample, at least 1000 binucleated cells were scored and micronuclei were recorded using an AxioImager A1 microscope (Carl Zeiss, Jena, Germany) with 400× or 1000× magnification.

2.5. Cell proliferation index

A cytokinesis-block proliferation index (CBPI) was calculated according to method of Surrales at al. [40] as follows: CBPI = MI + 2MII + 3(MIII + MIV)/N, where MI–MIV represent the number of cells with one to four nuclei, respectively, and N is the number of cells scored.

2.6. Statistical analysis

Statistical analysis was performed using the statistical software package Statistica 8.0, and OriginPro 8 for Microsoft Windows. Statistical analysis was done using Student's *t* test and product–moment and partial correlations. P values less than 0.05 were considered significant and less than 0.001 were considered highly significant. Results are expressed as percentage of control.

2.7. Instrumentation

The absorption spectra were measured using the GBC Cintra 10 and Perkin Elmer Lambda 35 spectrophotometers with thermostated 1.00 cm quartz cell, in the wavelength range from 200 to 500 nm. The micronuclei were recorded using an AxioImager A1 microscope (Carl Zeiss, Germany) with 400× or 1000× magnification. pH values of the solutions were measured by a Methronom pH meter, Model 713.

3. Results

3.1. Effect of gold(III) complexes on Na^+/K^+ ATPase activity

The effect of H[$AuCl_4$], [$AuCl_2(DMSO)_2$]Cl and [$AuCl_2$bipy]Cl on the ATP hydrolysis catalyzed by Na^+/K^+ ATPase was investigated after addition of the complexes to the reaction mixture in the concentration range from 1×10^{-8} to 1×10^{-4} M. The results indicate that the complexes inhibit Na^+/K^+ ATPase activity in a concentration-dependent manner. The dependence of relative enzyme activity (REA), expressed as a percentage of the control value (Na^+/K^+ ATPase activity obtained without inhibitor), on the concentration of Au(III) complexes fits a sigmoid function (Eq. (1), Fig. 2).

$$y = \frac{A_1 - A_2}{1 + (x - x_0)^p} + A_2 \quad (1)$$

where x is inhibitor concentration in M, x_0 is equal to IC_{50} value (see below) and y is REA. A1, A2 and p are constant parameters ($A_1 \approx 100$, $A_2 \approx 0$, p is equal to Hill coefficient value, n (Table 1)).

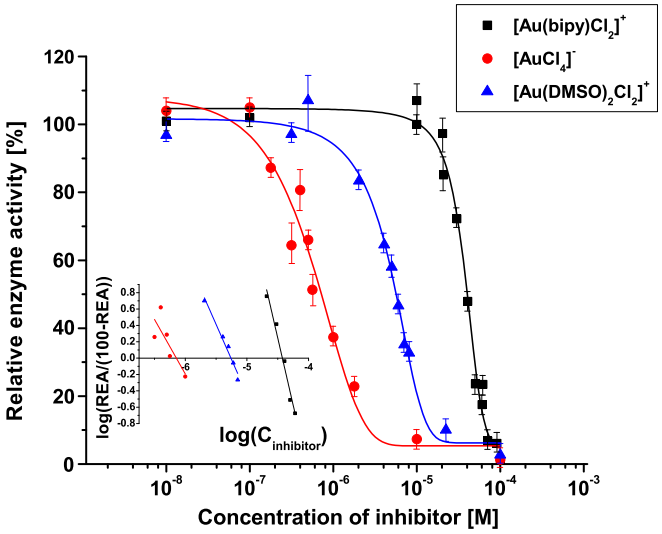

Fig. 2. Inhibiton of Na^+/K^+ ATPase with Au(III) complexes. Inset: Hill analysis of inhibition curves.

The concentration range of inhibitors relative to the linear region of the sigmoid plot was used for Hill analysis according to Eq. (2) (Fig. 2 (inset)).

$$\log\left(\frac{REA}{100 - REA}\right) = -n\log[I] + n\log IC_{50} \quad (2)$$

where, [I] is complex concentration, IC_{50} is half maximum inhibitory activity (*i.e.* the concentration of the complex with the ability to inhibit 50% of Na^+/K^+ ATPase activity after some exposure time) and n is Hill coefficient. The values of inhibition parameters (IC_{50} and n) determined by sigmoid fitting the experimental data and Hill analysis are given in Table 1. The obtained results suggest a significant difference in inhibitory potency of the investigated complexes toward Na^+/K^+ ATPase activity. Thus, H[$AuCl_4$] ($IC_{50} = (5.75 \pm 0.02) \times 10^{-7}$ M) exhibits ten times stronger inhibition than [$AuCl_2(DMSO)_2$]Cl ($IC_{50} = (5.50 \pm 0.02) \times 10^{-6}$ M), while Na^+/K^+ ATPase is a hundred times less sensitive toward [$AuCl_2$bipy]Cl ($IC_{50} = (3.98 \pm 0.02) \times 10^{-5}$ M) compared to H[$AuCl_4$]. Hill coefficient values (Table 1) above 1 ($n > 1$) indicate a positive cooperation for all complexes binding to Na^+/K^+ ATPase.

3.2. Prevention and recovery of gold(III) complexes induced Na^+/K^+ ATPase inhibition by L-Cys and gluthatione

Two series of experiments were performed to elucidate the effect of –SH containing ligands, L-Cys and GSH, on the inhibition of Na^+/K^+ ATPase activity. In the first experiment, the enzyme was preincubated with varying concentrations (from 1×10^{-6} to 1×10^{-3} M) of L-Cys or GSH for 15 min at 37 °C. Afterwards, the Au(III) complexes were added in the concentrations that cause 20% (IC_{20}), 50% (IC_{50}) and almost complete (IC_{100}) decreasing the Na^+/K^+ ATPase activity (see Fig. 2), and kept in contact with the enzyme and the ligand for 10 min. The dependence of the relative enzymatic activity *vs.* the concentration of

Table 1
IC_{50} and Hill coefficient (n) values for inhibition of Na^+/K^+ ATPase induced by Au(III) complexes.

Complex	Sigmoid fit	Hill analysis	
	IC_{50} [M]	IC_{50} [M]	n
$[AuCl_4]^-$	$(7.19 \pm 0.02) \times 10^{-7}$	$(7.24 \pm 0.02) \times 10^{-7}$	5.26 ± 0.01
$[Au(DMSO)_2Cl_2]^+$	$(5.50 \pm 0.02) \times 10^{-6}$	$(5.49 \pm 0.02) \times 10^{-7}$	2.25 ± 0.01
$[Au(bipy)Cl_2]^+$	$(3.88 \pm 0.02) \times 10^{-5}$	$(3.84 \pm 0.02) \times 10^{-6}$	4.52 ± 0.01

the –SH containing ligands in the presence of IC_{20}, IC_{50} and IC_{100} of complexes is presented in Fig. 3. The results show that there is a dose-dependent prevention of enzyme inhibition in the presence of L-Cys or GSH. It is obvious from Fig. 3 that the 1 mM ligand concentration results in the marked prevention of the enzyme inhibition. In the case of GSH, even 0.1 mM induces a significant increase in the remaining enzyme activity (about 40%) in the presence of IC_{100} complexes. This fact indicates a higher capability of this –SH containing ligand, compared with L-Cys, to prevent Na^+/K^+ ATPase inhibition by Au(III) complexes. Approximately complete prevention was only obtained by the action of 1 mM GSH or L-Cys at IC_{20} of each of three complexes.

The results obtained for the described prevention by the –SH containing ligands showed that it was reasonable to investigate the ability of L-Cys and GSH to recover Na^+/K^+ ATPase activity after the inhibition induced by the appropriate Au(III) complexes concentrations. In another series of experiments, L-Cys or GSH, in the concentration range from 1×10^{-6} to 1×10^{-3} M, was added into the incubation mixture 10 min after the enzyme had been exposed to IC_{20}, IC_{50} or IC_{100} of each inhibitor (at 37 °C). This preincubation period was long enough to establish the equilibrium between the complexes and enzyme. After 15 min contact between the ligand and formed enzyme–inhibitor complex, the enzyme reaction was started by ATP addition. The results presented in Fig. 4 display that the applied thiols possess a dose-dependent recovery effect on the diminished Na^+/K^+ ATPase activity exposed to the inhibiting

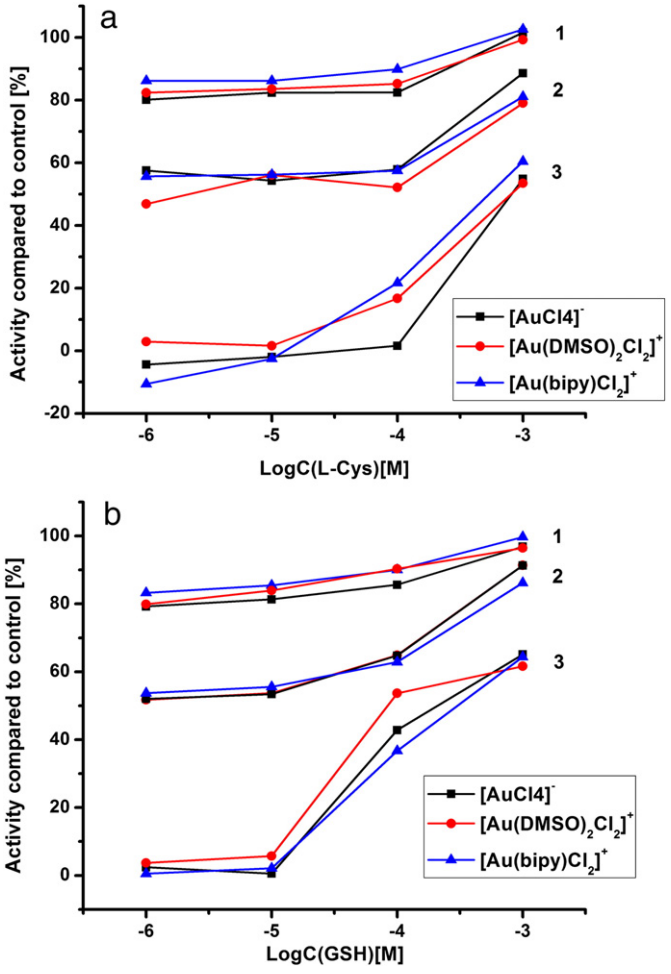

Fig. 3. Preventive effects of L-Cys (a) and GSH (b) on Na^+/K^+ ATPase activity in the presence of Au(III) complexes at concentrations which induce 20% (line group 1), 50% (line group 2) and 100% (line group 3) enzyme inhibition.

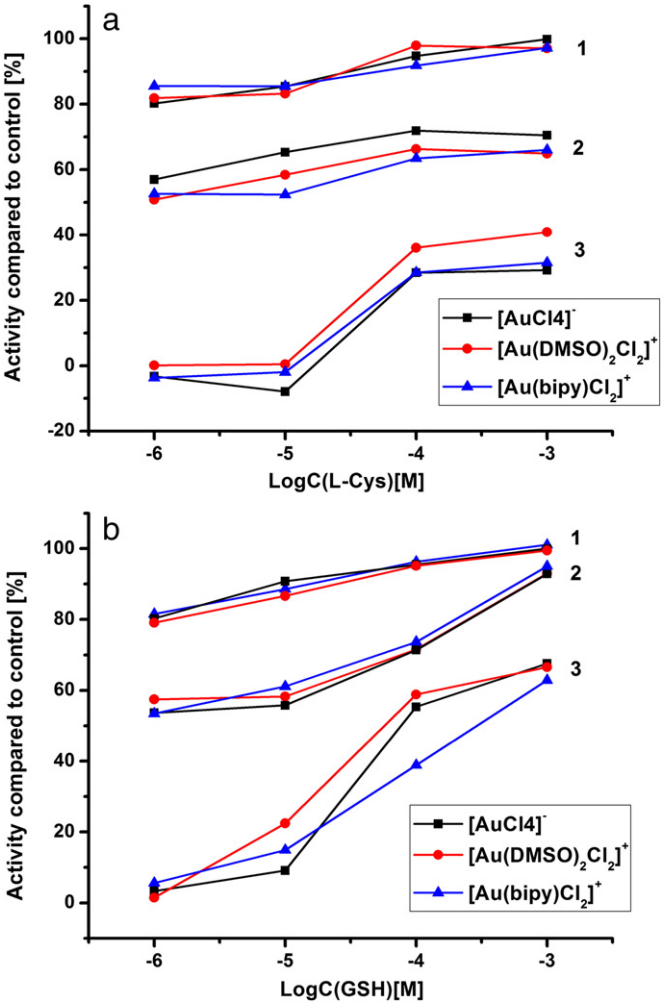

Fig. 4. Effects of L-Cys (a) and GSH (b) on the reactivation of the Na^+/K^+ ATPase activity inhibited in the presence of Au(III) complexes at concentrations which induce 20% (line group 1), 50% (line group 2) and 100% (line group 3) enzyme inhibition.

concentrations of complexes. Similar to the prevention of the inhibition, the ligand concentration of 1 mM leads to the noticeable regeneration of the decreased enzyme activity induced by the investigated Au(III) complexes (IC_{20}, IC_{50} and IC_{100}). Additionally, GSH demonstrates a stronger recovery capability than L-Cys, at higher concentrations, for IC_{50} and IC_{100} (Fig. 4, line groups 2 and 3). Also, 0.1 mM L-Cys addition, as well as GSH, significantly recovers the total enzyme inactivation caused by each of the inhibitors (Fig. 4, line group 3). The (approximately) complete regeneration is only possible at less enzyme inactivation (20% inhibited activity) using higher concentrations of either reactivators (Fig. 4, line group 1).

3.3. Kinetic analysis

The detailed kinetic analysis of Na^+/K^+ ATPase inhibition induced by the investigated complexes was performed in order to determine the mode of interaction between the enzyme and inhibitors. For this purpose, the initial reaction rate (v_o) in the presence and absence of Au(III) complexes was determined as the function of $MgATP^{2-}$ in the concentration range from 0.15 to 5 mM, while maintaining the concentrations of other substances in the reaction mixture constant. The inhibitor concentrations were selected from the inhibition curves (Fig. 2), as the concentrations that inhibited the enzyme activity were about 50%. These were 5×10^{-5} M for $[Au(bipy)Cl_2]^+$, 5×10^{-6} M

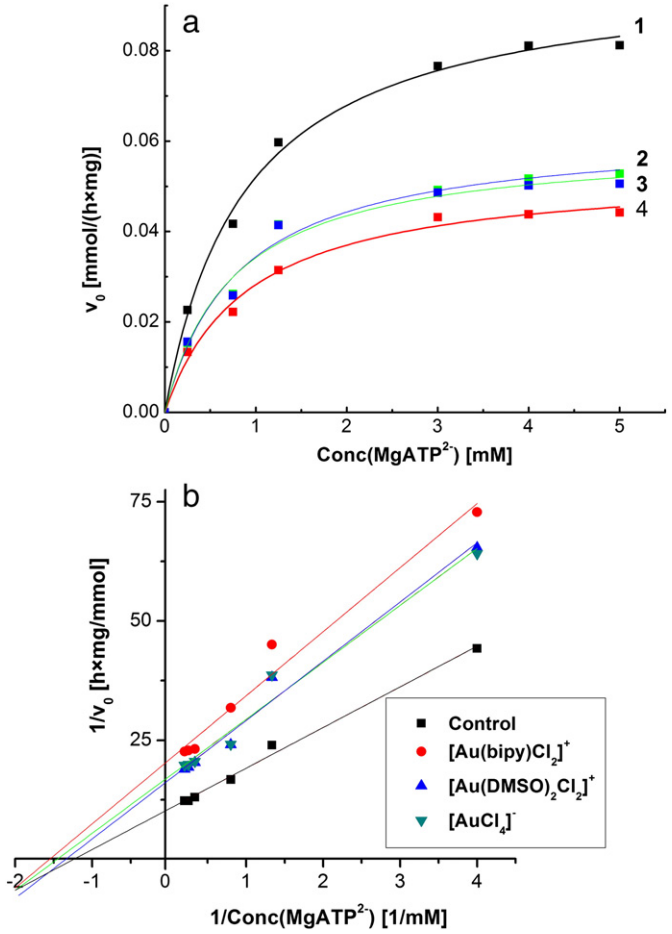

Fig. 5. a) Initial reaction rate (v_o) vs. $MgATP^{2-}$ concentration in the absence (1) and presence of 5×10^{-6} M for $[Au(DMSO)_2Cl_2]^+$ (2), 5×10^{-5} M $[Au(bipy)Cl_2]^+$ (3) and 5×10^{-7} M $[AuCl_4]^-$ (4). The values given are the mean of at least three experiments ± S.E.M. b) The Lineweaver–Burk transformation of the data.

for $[Au(DMSO)_2Cl_2]^+$ and 5×10^{-7} M for $[AuCl_4]^-$. The obtained results fit the typical hyperbolic Michaelis–Menten curve (Fig. 5a).

Kinetic parameters, K_M and V_{max}, were calculated from hyperbola function (Fig. 5a), as well as using linear Lineweaver–Burk transformation. The dependences of $1/v_o$ *vs.* $1/C_{MgATP}^{2-}$ were presented and linear plots were obtained in all cases (Fig. 5b). The values of K_M and V_{max} were evaluated from the intercept of the linear plots with x and y axes, ($1/K_M$ and $1/V_{max}$, respectively). The results are summarized in Table 2. V_{max} decreased in the presence of the inhibitors in all cases, while K_M remained constant, compared to the control sample without the inhibitor (Table 2). The obtained values suggest the noncompetitive reversible type of Na^+/K^+ ATPase inhibition by the used Au(III) complexes, since the K_M value was not affected by the inhibitor binding, while V_{max} decreased in the presence of each of the complexes.

Table 2
The values of K_M and V_{max} determined from Michaelis–Menten hyperbola function and its linear Lineweaver–Burk transformation.

Complex	K_M [mM]	V_{max} [mmol/h/mg]
Control	0.79 ± 0.07	0.094 ± 0.005
$[AuCl_4]^-$	0.63 ± 0.10	0.047 ± 0.004
$[Au(DMSO)_2Cl_2]^+$	0.73 ± 0.11	0.059 ± 0.005
$[Au(bipy)Cl_2]^+$	0.67 ± 0.11	0.057 ± 0.005

3.4. Influence of gold(III) complexes on human lymphocytes

The effects of increasing concentrations of Au(III) complexes on the incidence of micronuclei and proliferation index in lymphocyte cultures are presented in Fig. 6.

In lymphocyte cultures, significant concentration-dependent increase ($p < 0.05$) of the incidence of micronuclei was observed in all samples treated with $[Au(bipy)Cl_2]^+$. At concentration range from 10^{-8} M–10^{-5} M, the incidence of micronuclei was enhanced by 10.41%–30.72% relative to control, while at the highest employed concentration (10^{-4} M) the 4-fold increase of the incidence of micronuclei was observed ($p < 0.001$). In lymphocyte cultures treated with $[AuCl_4]^-$ micronuclei formation slightly increased in a dose-dependent manner up to concentration of 10^{-7} M, afterwards it significantly ($p < 0.05$) decreased compared to control (by 34.47%–49.91%). Similar reduction in micronuclei formation (by approximately 50%, $p < 0.001$) was observed in all samples treated with increasing concentrations of $[Au(DMSO)_2Cl_2]^+$.

Concentration-dependent, yet insignificant increase of CBPI was observed in samples treated with all the investigated Au complexes. However, at the highest employed concentration (10^{-4} M) of complex $[Au(bipy)Cl_2]^+$, the significant suppression of cell proliferation was observed ($p < 0.05$). The incidence of micronuclei and cytokinesis-block proliferation index correlated inversely, and was statistically significant ($r = -0.98$, $p < 0.05$). In samples treated with $[Au(DMSO)_2Cl_2]^+$ and $[AuCl_4]^-$, no significant correlation between micronuclei induction and cell proliferation index was observed.

4. Discussion

In this study we explored the influence of three selected Au(III) complexes on the activity of commercially available porcine cerebral cortex Na^+/K^+ ATPase. The obtained results (Fig. 2) demonstrate a concentration-dependent inhibitory effect of all the explored compounds on the enzyme activity, but with variable potencies. These results are in agreement with previous studies that have already demonstrated the affinity of gold and similar transition and heavy metals (palladium, platinum *etc.*) [33,34,41] to react with –SH groups of proteins. For all studied Au(III) complexes the action of gold is more complex than simple interaction with the –SH groups, and the redox reactions with L-Cys residues and disulfide bonds of Na^+/K^+ ATPase must be also taken into account. The disulfide bridges in the Na^+/K^+ ATPase are required for enzyme functionality. The removal of disulfide bonds from the ß subunit due to the redox reactions of gold complexes can lead to the significant functional alterations [42]. Moreover, the reduction of complexes with the tendency $[AuCl_4]^- > [Au(DMSO)_2Cl_2]^+ > [Au(bipy)_2Cl_2]^+$ can occur and the two oxidation states of gold may behave differentially toward the enzyme [43]. It is interesting to note here that a gradient in the tendency towards reduction correlates with the potency of inhibitors, but we believe that it is only one of the contributing factors in the process. Stability of the complex and size of the ligand also affect the potency of an inhibitor. In all cases the high Hill coefficient is observed. This suggests a strong positive cooperation of the inhibitor binding to the enzyme. In essence, after the first molecule of the inhibitor has reacted with the enzyme, other molecules bind even easier.

The prevention of inhibition and reactivation of the inhibited enzyme have been achieved using potent –SH donors. The likely reason for prevention of inhibition is the fast formation of the inactive $[AuCl(L\text{-}Cys)(DMSO)_2]^+$ complex analogous to the $[PtCl(L\text{-}Cys)(DMSO)_2]$ of Bugarčić et al. [44], prior to the formation of the [Au(III)complex (enzyme)]$^+$ complex. Further reduction of Au(III), resulting in the formation of Au(I) and Au colloids in the slower second reaction step, is likely in the prevention and the recovery experiments when GSH and L-Cys were added to the media. This process is common for –SH

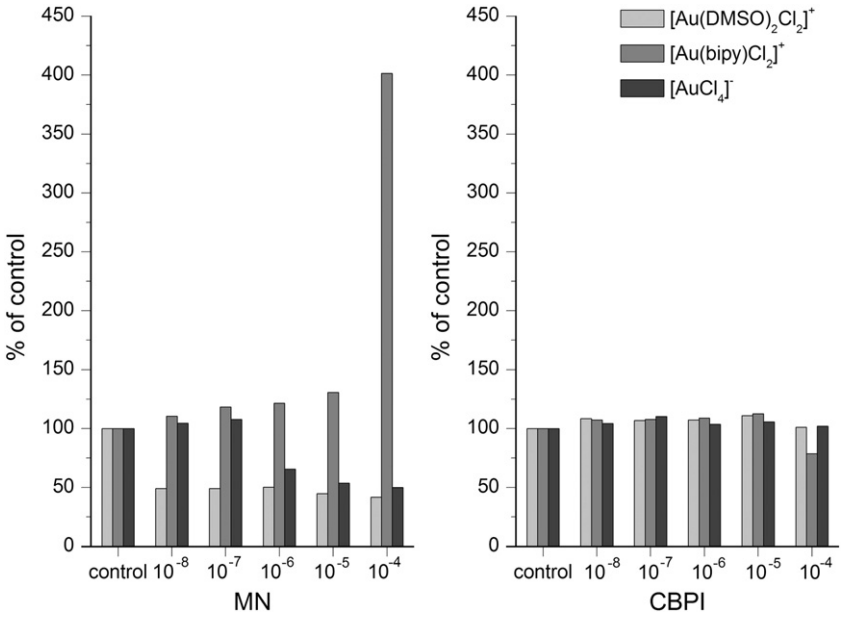

Fig. 6. Incidence of micronuclei (MN) and cytochalasine B proliferation index (CBPI) (expressed as percentage of control) in lymphocyte cultures treated with increasing concentrations of Au(III) complexes.

containing ligands, and the fast replacement of the ligand in coordination sphere of Au(III) complexes leads usually to reduction [16]. This reduction leads also to the formation of colloidal Au particles which are even able to enhance the enzyme activity [31]. The recovery of the activity was achieved when the concentration of thiols was equal or higher than the concentration of the complexes. These results can be explained by enzyme extruding from [Au(III)complex(enzyme)] complex and its substitution with smaller and more reactive –SH donor. A similar effect was earlier demonstrated on Pd(II) complexes [34]. In addition, Au(III) compounds can oxidatively cleave disulfide bonds of β unit, and this may contribute to the lost non-recoverable enzyme activity [42,45,46].

Kinetic studies have shown that the inhibition is a noncompetitive type, and that K_M remains constant after the inhibition. This gives some insights on the mechanism of inhibition, suggesting that it likely involves binding to the –SH groups that are not in the ATP binding site of the enzyme. The investigated Na^+/K^+ ATPase in these experiments is a membrane enzyme, purified from porcine cerebral cortex. In such prepared plasma membrane fragments, these –SH groups are probably not encapsulated, and they are accessible to the Au(III) compounds. For that reason, the obtained *in vitro* results would be different from the potential interactions of the Au(III) compounds and Na^+/K^+ ATPase using more complex model systems such as synaptosomes, cell cultures, experimental animals. Since the enzyme has around 64 different –SH groups, it would be difficult to determine the precise amino acid residues that are at the inhibitor binding sites. These results are in agreement with previous studies [34].

Biological investigations were performed on human lymphocytes *in vitro* employing cytokinesis-block micronucleus assay designed by Fenech [39]. The test measures DNA damage, cytostasis and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated (BN) cells who display small round bodies-micronuclei (MN). Micronuclei arise from acentric chromosome structures (misrepaired DNA damages) or whole chromosome which was not carried to the opposite poles during the anaphase. Their formation results in the daughter cell lacking a part or all of a chromosome. Incidence of micronuclei serves as an index of genetic damage. Cytostatic effects are measured by proportion of mono–bi- and multinucleated cells-cytochalasin B proliferation index (CBPI). These parameters correspond to the amount of DNA damage in a living cell and to the cytotoxicity of the tested complex, respectively. Except for a modest increase of proliferative capacity of cells, which might suggest disturbed function of cell cycle checkpoints, the complexes $H[AuCl_4]$ and $[Au(DMSO)_2Cl_2]Cl$ did not induce cytotoxic effects in human lymphocytes. However, extensive and dose-dependent damage to DNA (observed as a rise in the incidence of micronuclei) was observed after treatment of the cells with the complex $[Au(bipy)Cl_2]Cl$, which caused cytotoxic effects as revealed by significant enhancement of micronuclei incidence and suppression of cell proliferation, particularly at the highest concentration employed. These results are in accordance with several studies that reported antiproliferative properties of Au(III) complexes [8,11,12]. Since current cancer therapy mostly relies on DNA damaging agents to induce antiproliferative effects in cancer cells, these results suggest the potential use of $[Au(bipy)Cl_2]Cl$ in antitumor therapy. However, potential occurrence of severe side-effects in cancer therapy using Au or other metal complexes should not be underestimated. Taken together, results of this study clearly indicate that the micronuclei induction and proliferative potential of lymphocytes strongly depend not only on concentration but also on the type of the complexes.

5. Conclusion

We conclude that the complexes we explored have shown a dose-dependent inhibition of the porcine brain cortex Na^+/K^+ ATPase. This inhibition is noncompetitive and it is achieved *via* the –SH groups of the enzyme. This inhibition can be prevented if –SH containing ligands such as L-Cys and glutathione are present in the reaction medium, and it can be partially reversed if the inhibited enzyme is treated with those same –SH containing ligands. The biological effects upon the living cells match those that are seen with other similar inhibitors — the cell's normal life cycle and DNA repair process is disrupted.

In summary, elucidation of the reaction parameters between Au(III) complexes and biomolecules is helpful in: a) estimation of potential toxicity of complexes that might be further developed and used in the tumor therapy and, thus for b) development of

approaches for prevention of side effects of the therapy. This prevention includes the administration of –SH containing molecules.

Abbreviations

bipy	2,2′-bipyridine
CBPI	cytochalasin B proliferation index
GSH	glutathione
MN	micronuclei
REA	relative enzyme activity

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INTERACTION OF GOLD NANOPARTICLES WITH RAT BRAIN SYNAPTOSOMAL PLASMA MEMBRANE Na^+/K^+- ATPase AND Mg^{2+}-ATPase

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The aim of the work was to investigate the interaction between borate capped gold nanoparticles (NPs) and the rat brain synaptosomal plasma membranes (SPM), as well as the effects of these NPs on SPM Na^+/K^+- ATPase and Mg^{2+}-ATPase activity. The changes in the UV-vis spectra of NPs and SPM assembly suggested the agglomeration and precipitation of NPs. FTIR measurements indicated that both protein –SH and $–NH_2$ groups and positively charged membrane fragments were implicated in the adhesion of SPM to the surface of NPs. AFM showed an increase in the particularization of the SPM material after mixing with gold NPs. Influence of gold NPs on Na^+/K^+-ATPase and Mg^{2+}-ATPase activity was investigated as the function of NPs and protein concentration, preincubation time and also in the presence of various concentrations of ouabain, the specific enzyme inhibitor. NPs induced the stimulation of Na^+/K^+-ATPase activity for more than 100%, since Mg^{2+}-ATPase activity reminded unaffected. We propose that this stimulation of enzyme activity was a consequence of an increase of the active surface of membranes.

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Keywords: Gold nanoparticles, Na^+/K^+-ATPase, Mg^{2+}-ATPase, synaptosomal plasma membranes, biofunctionalization.

1. Introduction

Gold nanoparticles (Au NPs) are a very attractive tool in biomedical research, because they have repeatedly shown great potential as substance carriers, active surfaces and even biologically active agents [1]. Since they can be readily taken up by cells [2], they are proposed in the medical sector as new tools in diagnostics [3] and drug delivery systems [4]. Although GNPs are considered being inert in biomedical applications [5], the size dependent cytotoxicity towards different cell types, with smaller particles being more toxic has been reported [6, 7].

The particles themselves are easily modified due to the gold's ability to bond with biologically important molecular groups such as amines, thiols, and carboxyl groups from amino acids and proteins [8, 9]. They have also been used as immobilization matrices for enzymes, where the immobilized enzyme was found to be more stable compared to free enzyme [10, 11]. Of all available molecular targets in living tissue, proteins have shown the greatest affinity towards GNPs. The conjugation of proteins with GNPs stabilized the system and also introduced biocompatibile functionalities into the nanoparticles for further biological application. [12-14] The binding of the proteins to nanoparticles might occur by electrostatic forces between the surface-terminated negatively charged groups capping the nanoparticle and the positively charged functional groups of the protein (e.g. aminoacids residues) [9, 15, 16].

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Na^+/K^+-ATPase and Mg^{2+}-ATPase (ecto-ATPase) are membrane enzymes ubiquitous in animal cells that involve 5'-adenosine triphosphate (ATP) as a substrate for their functioning. Na^+/K^+-ATPase plays a key role in the active transport of monovalent cations (Na^+ and K^+) across the membrane [17]. Beside its transporter function, Na^+/K^+-ATPase acts as the receptor for cardiac glycosides such as ouabain like compounds [18], which are the specific inhibitors of the enzyme. The enzyme is composed of α-subunit, which contains the adenosinetriphosphate (ATP), Na^+, K^+ and ouabain binding sites, as well as the site for phosphorylation and β-subunit, which stabilizes the K^+ binding cage. The ouabain insensitive ecto-adenosine triphosphatase (Mg^{2+}-ATPase) represents an integral membrane protein that, in the presence of divalent cations (Ca^{2+} or Mg^{2+}), hydrolyses extracellular nucleotides because of the outward orientation of its active site [19]. It is much less well characterized than sodium pump, but apparently consists of at least two forms with different molecular weights and sensitivity to metal ions. There is a great number of organic molecules and metallic ions that can modulate the activity of these enzymes [20, 21]. Literature surveys suggest that some NPs (Cu, TiO_2) inhibit Na^+/K^+ ATPase activity and induce oxidative stress [22, 23]. Also, it was shown that some of the gold compounds have an inhibitory effect upon enzyme [24]. On the contrary, the literature data indicate that gold and silver nanoparticles stimulated ATPases activity of native and rehydrated cells of *Escherichia coli* [25]. In general, there are some fundamental differences between the physiological effects of metal ions and NPs. The chemistry and behavior of metal nanoparticles involve dynamic aspects of aggregation theory, rather than equilibrium models traditionally used for free metal ions.

One of the available model systems for the study of Na^+/K^+-ATPase and Mg^{2+}-ATPase are the rat brain synaptosomal plasma membrane fragments (SPM) [26]. In this paper we describe the influence of borate capped Au NPs of various sizes on SPM Na^+/K^+-ATPase and Mg^{2+}-ATPase activity in this model system. In addition, the interaction of SPM with Au NPs was characterized by application of various techniques, such as UV-Vis spectrophotometry, Fourier transformation infrared spectroscopy (FTIR) and atomic force microscopy (AFM).

2. Experimental

2.1 Materials

Gold (III) chloride trihydrate ($HAuCl_4 \times 3H_2O$), sodium borohydride ($NaBH_4$, 99%), perchloric acid ($HClO_4$), ouabain, all from Aldrich, were used as received. Rat SPM were isolated according to a previously described method and stored at -80° C until use [26]. Adenosinetriphosphate (ATP), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), tris(hydroxymethyl) aminomethane (TrisHCl), were purchased from Sigma Aldrich (Germany). Stannous chloride ($SnCl_2$) and ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}$) were from Merck (Darmstadt, Germany). Water purified with a Millipore Milli-Q water system was used for preparing all solutions.

2.2 Preparation of gold nanoparticles

Au NPs were obtained by the reduction of $HAuCl_4$ with $NaBH_4$. Precisely, 100 mL of 0.2 mM $HAuCl_4$ was reduced by 5.3 mM of $NaBH_4$ at room temperature, to yield a ruby-red solution. The calculated value of NPs concentration was 2.8×10^{-9} M, assuming that the reduction from gold(III) to gold atoms was 100% complete.

2.3 ATPase activity measurements

The incubation mixture contained 400 mM NaCl; 80 mM KCl; 20 mM $MgCl_2$, 200 mM Tris HCl, pH=7.4 and 25μL of SPM containing 1 mg/mL protein in total volume 200 μL at 37°C. 20 μL of 20 mM ATP was added to initiate the enzymatic reaction which was allowed to proceed for 15 min. Reaction was stopped by an addition of 22 μL of 3 M $HClO_4$ and by cooling of samples on ice. The concentration of liberated inorganic orthophosphate was determined

spectrophotometrically [26]. The activity obtained without NaCl and KCl in the medium assay was attributed to Mg^{2+}-ATPase. Na^+/K^+-ATPase activity was calculated as a difference between the total ATPase and Mg^{2+}-ATPase activity. The results represent the mean values of at least two experiments done in duplicate.

2.4 Apparatus

Transmission electron microscope (TEM, JOEL 100CX) was used to observe the morphology and average size of Au nanoparticles.

All spectrophotometric measurements were performed on a Perkin Elmer Lambda 35 UV-vis spectrophotometer with 1 cm path length quartz cuvette.

FTIR spectra were recorded on Thermo Electron Corporation Nicolet 380 FTIR Spectrophotometer with ATR (Attenuated Total Reflection) accessory, equipped with a diamond tip, in the region between 4000 and 500 cm^{-1}.

AFM measurements were carried out using commercial AFM VECCO Quadrexed Multi Mmode IIIE equipment operating in tapping mode (TMAFM) on highly oriented pyrolytic graphite (HOPG). Surface topography and phase images were simultaneously aquired using a commercial NanoScience-Team Nanotec GmbH SCN (Solid Nitride Cone) AFM probe, with tip radius lower than 10 nm. AFM observations were repeated on different areas from $2x2\mu m^2$ to $250x250nm^2$ on the same substrate at ambient laboratory conditions (about 20^oC)

Droplet-evaporation method was used for preparing AFM samples for analysis. A droplet of $2x10^{-9}$ M Au colloid or SPM containing 1 mg/mL of protein was deposited on freshly cleaned HOPG 1x1 cm. The droplet was then carefully washed after allowing the sample to sit about 15 minutes. For gold nanoparticles functionalized with protein, 100 µL of Au colloid was mixed with 25 µL of SPM. 15 min after mixing the sample was put on HOPG as described above.

3. Results

3.1 Spectrophotometric characterization of Au NPs interaction with SPM

The Au colloidal dispersion was used to investigate the interaction with SPM. According to TEM measurements (data not shown), the nanoparticles were spherical in shape with the average particle diameter 9.5±0.8 nm.

UV-vis spectroscopy was initially used to monitor spectral changes in colloidal solutions upon addition of SPM. The spectra were recorded before and after the mixing of 100 µl of SPM and 25µl of colloid solution containing $2x10^{-9}$ M NPs, under stirring at room temperature. This was then diluted with water to 1.5 mL and measured. The gold NPs show an intense surface plasmon resonance (SPR) band at 518 nm due to the collective excitation of conducting electrons in a metal (Fig. 1 curve 2).

Adding SPM into the gold colloidal solution, some spectral changes were observed. (Fig. 1 curves 3-5). The spectral changes of SPR band resulted from the replacement of borate ions on the NPs surface by the SPM molecules that caused a significant decrease in the stability ratio and aggregation of NPs. The agglomeration was extremely fast and fine precipitates were formed after 10 min of mixing with SPM. As a consequence of agglomeration, the corresponding SPR band was broadened and shifted to longer wavelengths due to dipole-dipole interactions of high induced dipole moment of NPs in aggregates. The SPR band completely disappeared after 20 min.

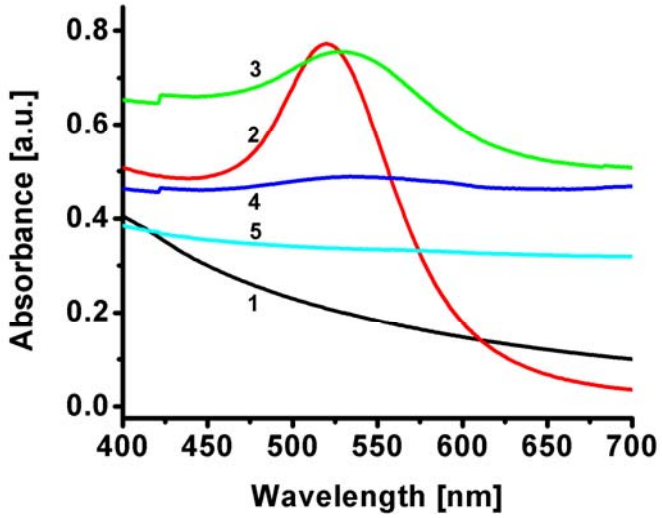

Fig. 1. UV-vis spectra of the solutions containing 66.6 µg/mL SPM (1) and 3.3x10^{-11} M Au NPs (2) and 1 min (3), 10 min (4) and 20 min (5) after the mixing.

3.2 FTIR characterization of Au NPs with SPM

FTIR spectra were recorded in order to evaluate the possible types of interactions that exist between the Au NPs and SPM. A number of vibration bands can be seen in the two different regions of the spectra as shown in Fig. 2. The FTIR spectrum of the SPM in water solution exhibits small bands located at 2940-2820 cm^{-1} related to the protein and lipid C-H asymmetric and symmetric stretching vibrations. The interaction Au NPs with SPM molecules leads to increase intensity and narrowing of the band at 2928 cm^{-1} due to the asymmetric stretching mode of the methylene groups in lipid sequences. Also, the rigid chain conformation on the NPs surface restricts protein mobility, resulting in the narrowing of the stretching bands [27, 28].

The multiply bonded CO group provides the intense bands between 2100 and 1900 cm^{-1}, clearly visible in the Fig.2. The new mode due to scissoring of a methylene group adjacent to the Au-S bond (δ_S) appears at 1430 cm^{-1} together with the weak bands around 1320 cm^{-1}. These bands are related to C-N, C-O vibrations of the protein backbone and amino acid residues and are shown in the spectrum. In addition, the weak band at 1050 cm^{-1} can be attributed to vibrations involving interaction between C-O stretching and C-N stretching. This band is shifted to 1160 cm^{-1}. Such effects are not surprising given the proximity of the gold surface to the bonds involved in these motions.

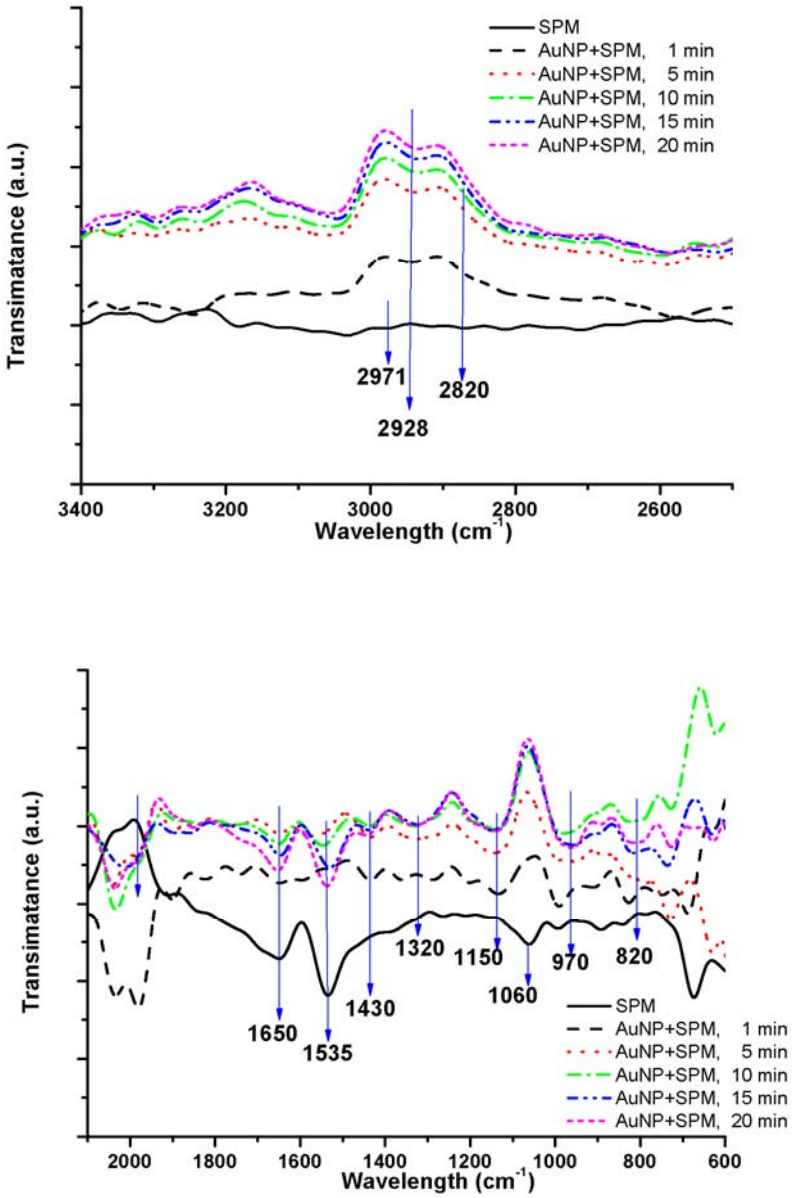

Fig. 2. FTIR spectra of SPM over time upon the addition of Au NPs

The specific region at 600-700 cm^{-1} assigned to the νS-C symmetric stretching vibration shows significant changes together with the bands at 970 and 820 cm^{-1} assigned to νAu-S symmetric stretching vibration, indicating that Au NPs and SPM interact via sulfur group [29]. The next characteristic vibration bands arise from the amide groups of proteins and provide information on its secondary structure, have been identified. Among them, amide I band (C=O stretching mode) appears at 1650 cm^{-1} and the amide II band due to the coupling C-N stretch to N-H bending at 1535 cm^{-1} [30]. The NPs interactions with SPM lead to increase intensity of these bands due to the interaction (via H-bonding) with protein C=O and C-N groups. Also, the slight displacement of the maximum amide I band from 1650 to 1652 cm^{-1} and amide II band from 1535 to 1537 cm^{-1} were observed upon addition of Au NPs.

3.3 AFM visualization of Au NPs interaction with SPM

The AFM measurements were performed with Au NPs in the absence and presence of SPM on HOPG. A representative AFM image of Au NPs in the absence of SPM is shown in Fig. 3. The section analysis of the image indicates the peaks, which correspond to the Au NPs anchored on the HOPG. The sizes of the particles were measured directly from AFM images. Single colloids display homogenous lateral and vertical dimensions. Height evolution was obtained by cross section profile in Fig. 3b. The size of nanoparticles (average diameter 9.4 nm) is in good agreement with the results obtained by TEM measurements.

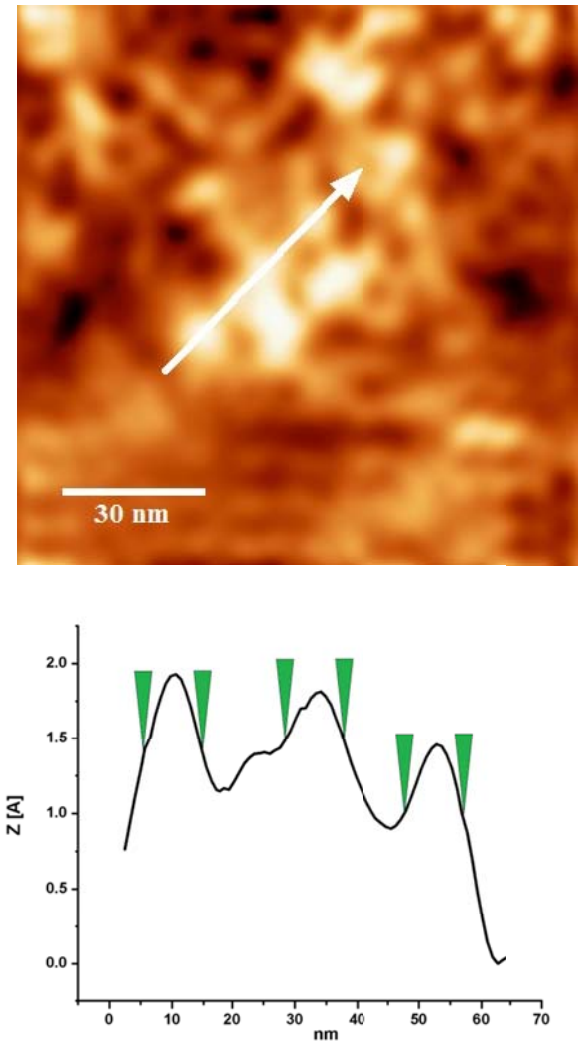

Fig. 3. AFM images of Au NPs deposited on HOPG after 15 min deposition time, scanned area 250x250 nm^2; a) 2D topography with cross section, b) cross section profile.

Fig. 4. shows the typical image of SPM membrane patches well distributed on the surface, in which the particles that are supposed to be membrane proteins are clearly observed. The stringy structure of SPM can be noticed.

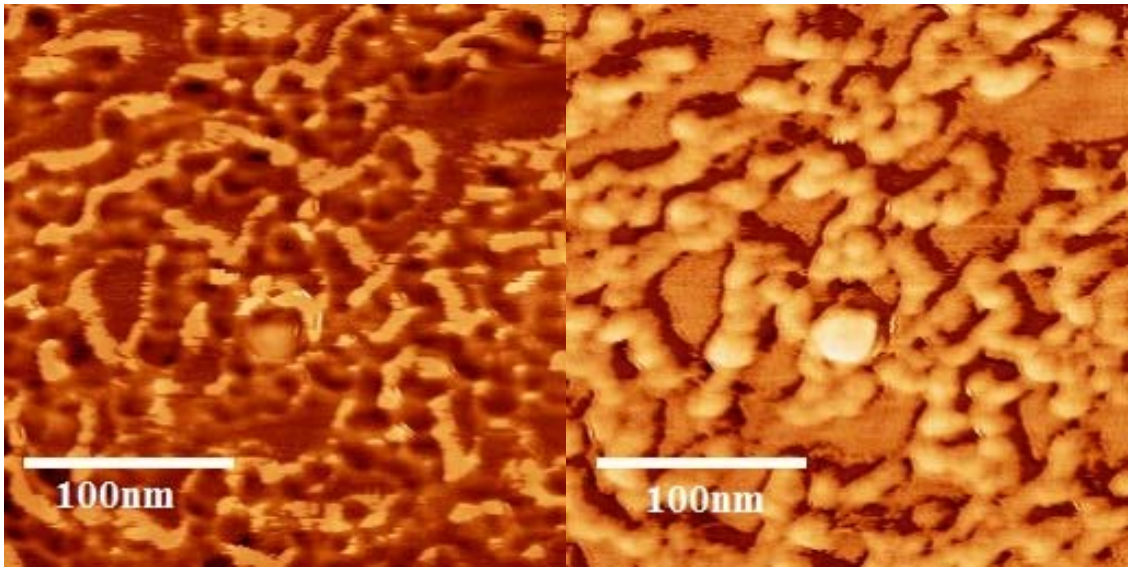

Fig. 4. AFM images of SPM deposited on HOPG after 15 min deposition time, scanned area 250x250 nm^2; a) - 2D topography, b) - 2D phase view.

Fig. 5. shows the mixture of SPM and Au NPs. It can be seen that there are no string-like structures and that most of the material is divided into smaller spherical aggregations. The results presented in Figs. 4 and 5 obtained from the AFM measurements indicated a change in the fine structure of SPM aggregates. A greater degree of particularization can be seen in SPM samples treated with colloid particles.

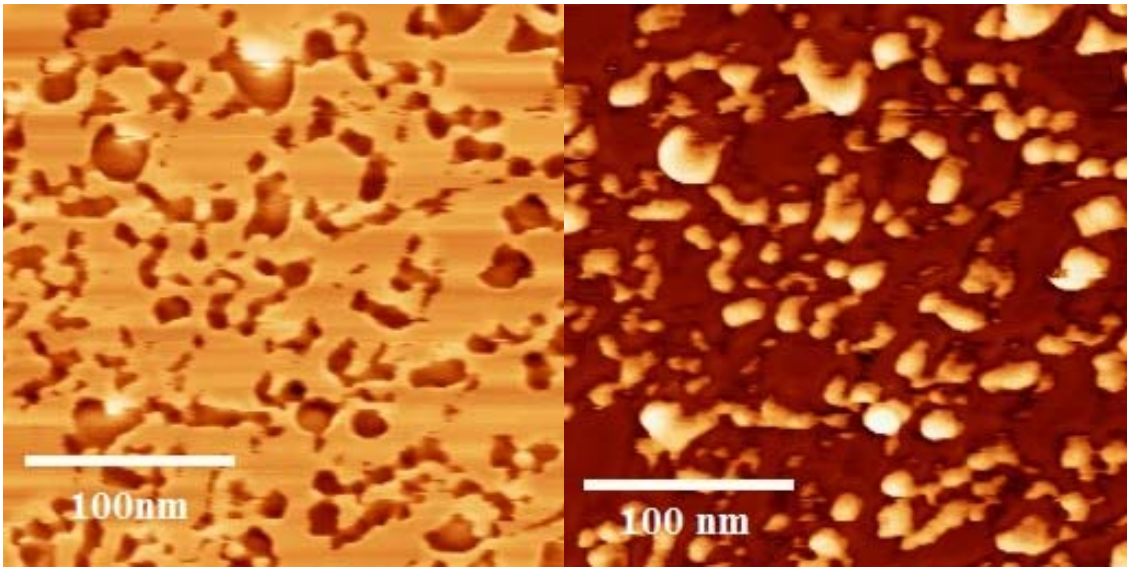

Fig. 5. AFM images of SPM treated with Au NPs deposited on HOPG after 15 min deposition time, scanned area 250x250 nm^2; a) - 2D topography, b) - 2D phase view.

3.4 Influence of Au NPs on ATPase activity

The influence of Au NPs within the concentration range from 10^{-10} – $5.25x10^{-10}$ M on the ATP hydrolysis catalyzed by SPM Na^+/K^+-ATPase and Mg^{2+}-ATPase was investigated, with the aim to find out if proteins retain their functionality in the presence of colloids. In this experiment the medium assay contained 20 μg protein. The catalytic reaction was started by addition of ATP

1 min after the nanoparticles were added into the medium assay and the results are presented in Fig.6.

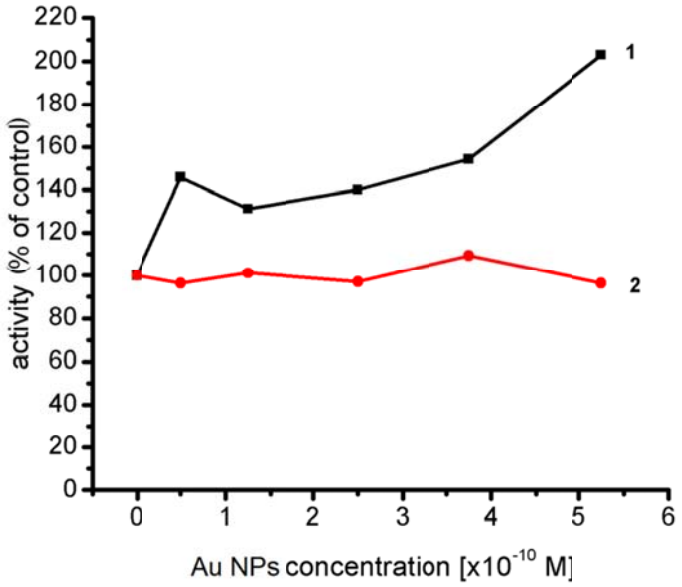

Fig. 6. Influence of Au NPs on Na^+/K^+-ATPase (1) and Mg^{2+}-ATPase (2) activity after 1 min preincubation.

The results indicated, that the borate capped Au NPs increased the activity of Na^+/K^+ -ATPase in the concentration dependent manner, compared to the control value without nanoparticles, since the activity of Mg^{2+}-ATPase remained constant. Moreover, the increse of the Na^+/K^+ -ATPase activity was more than 100% in the presence of $5.25x10^{-10}$ M Au NPs.

In the additional experiment the incubation time of SPM with nanoparticles (concentration was $5.25x10^{-10}$ M) was varied from 0.5 min to 120 min. The Na^+/K^+-ATPase activity increased for about the same value (100%) in all cases compared with the control value without nanoparticles, since Mg^{2+}-ATPase activity reminded unchanged.

Furthermore, an experiment with a constant concentration of nanoparticles (5.3×10^{-10}M) and a varying the amount of protein (from 5 μg – 40 μg total amount in medium assay) was carried out in order to investigate the trend of activity rise that was shown for Au NPs. The increase of activity for 85 – 110% compared to the control probe containing the same protein concentration without Au NPs was obtained in all cases.

In order to see if the interaction between Au NPs (concentration $5.25x10^{-10}$ M) and SPM Na^+/K^+-ATPase altered the enzyme selectivity towards its specific inhibitor, a set of activity measurements was carried out in the presence of ouabain, a selective inhibitor of Na^+/K^+-ATPase. Concentration span of ouabain in the assay was from $1x10^{-8}$ M to 10^{-5} M. Simultaneously, the inhibition was investigated in the medium assay of the same composition without Au NPs. Results show that the response of the enzymatic activity to the ouabain concentration is biphasic, indicating high and low affinity Na^+/K^+-ATPase isoforms (Fig.7). It is clear from the experimental results that the activity vs. ouabain concentration plots in both cases can be represented by the sum of two overlapping sigmoid curves, separated by a plateau as found in our previous work. [26, 31], [32]

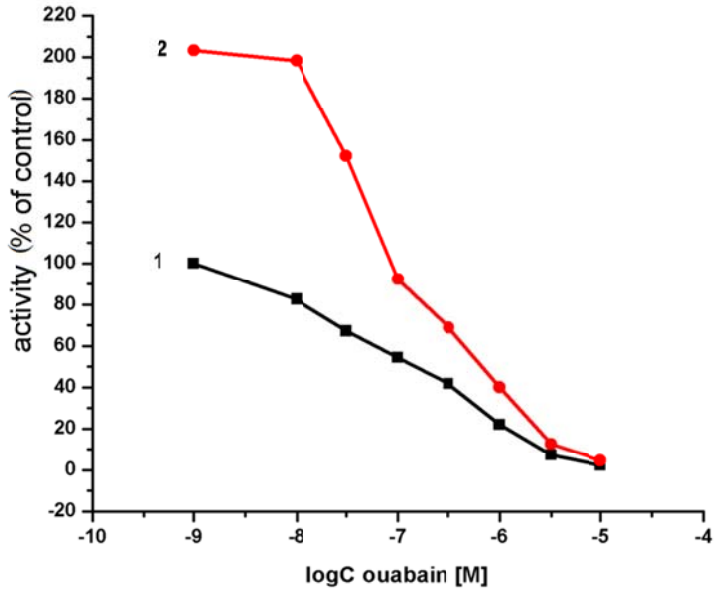

Fig 7. Inhibition of Na^+/K^+-ATPase activity by ouabain in the presence (1) and absence (2) of $5.25x10^{-10}$ M Au NPs.

The concentration dependent inhibition curves which correspond to high and low affinity inhibitor sites were obtained can be presented as the superposition of two sigmoid curves. Our results show that there was no major change in the IC_{50} values of ouabain. IC_{50} was found to be $(5.29\pm0.29)\cdot10^{-7}$ M for high and $(2.36\pm0.29)\cdot10^{-6}$ M for low affinity binding sites in both cases.

4. Discussion

The position of SPR band of Au NPs is affected by the particle size, shape, inter-particle distance and local dielectric environment of the particles [33]. Since the prepared Au NPs possessed a negative charge due to the adsorbed borate ions, the repulsive forces worked along particles and prevented their aggregation. Previous studies on nanoparticle-protein interactions suggest that adsorption of protein on the gold surface can induce formation of aggregates as a consequence of interaction between the positive surface residue of protein and the negative charge surface layer so the protein molecules constitute bridge among Au NPs [12, 34]. Also, during the formation of an adsorption layer on the Au surface, the anions can be replaced by protein upon adsorption, with the amino acids functional groups (amine and thiol groups) that interact directly with the Au surface [35-38]. Furthermore, the hydrophobic interactions among protein molecules within the protein layer adsorbed on the gold surface cannot be excluded. In the case of Au NPs, where borate anions could be easily replaced by the SPM molecules, a direct binding to the particle surface occurs *via* thiolate (–SH group) linkages through the cysteine residues [39], rather then $–NH_2$, which is consistent with the greater affinity of Au for –SH compared to $–NH_2$.

Consequently, these interactions can induce changes in the protein structure like a conformation changes, unfolds in protein near the particles surface, etc. The positions of amide I and amide II bands in the FTIR spectra of proteins are sensitive indicator of conformational changes in the protein secondary structure [30]. The amide I band is the sum of overlapping component bands (α-helix, β-sheet, β-turn and randomly coiled conformation), influenced by their different environment [40], while the amide II band indicates the amount of protein adsorbed on surface. The NPs interactions with SPM lead to the slight displacement and increase intensity of these bands. A probable reason for these changes is the orientation effects of the SPM molecule

during the interaction with gold surface. Nonetheless, it is worth noticing that no major rearrangement of the protein secondary structure upon interaction with gold surface occured.

AFM measurements enabled us to get information on the topography of membrane-bound proteins with extramembraneous protrusions. The lower domains can be described as the double lipid layer, since the higher domain represents the membrane parts with the high concentration of the transport protein [41],[42]. The results obtained by AFM measurements could imply that the presence of colloid particles caused further membrane fragmentation on a nano scale, resulting in greater effective membrane surface which in turn enables more Na^+/K^+-ATPase molecules to hydrolyze ATP, thus creating a stimulation of enzyme activity. It increased either by increasing local enzyme concentration on the particle surface, or simply by revealing more active centers. We also propose that the rise in the activity of SPM Na^+/K^+-ATPase may be a consequence of the immobilization of plasma membrane fragments on the surface of the particles. Also, the small surface of the particle in Au NPs might cause the enzyme units to cooperate or perhaps create a local rise in the concentration of substrate and/or cofactors (Mg^{2+}, Na^+ and K^+ ions) necessary for the function of the enzyme, thus facilitating its operation.

5. Conclusion

Spectrophotometric, FTIR and AFM analysis of borate capped Au NPs – SPM assembly indicated that interactions between NPs and proteine molecules occured due to the chemical bonding between NPs surface and positive charged cysteine moiety. The selectivity of the enzyme towards its natural inhibitor, ouabain, was preserved and unchanged. We propose that this rises in enzyme activity (observed through biochemical assays) is a result of chemical bonding and spatial reorganization of SPM on the colloid surface (observed through spectroscopic methods) which induced physical fragmentation and improved distribution of SPM aggregates across the colloid particles (seen on AFM).

Acknowledgements

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In vitro effects of some gold complexes on Na^+/K^+ ATPase activity and cell proliferation

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ABSTRACT

The *in vitro* influence of gold(III) complexes, $H[AuCl_4]$, $[Au(DMSO)_2Cl_2]Cl$ and $[Au(bipy)Cl_2]Cl$ (bipy = 2,2′-bipyridine), upon commercially available Na^+/K^+ ATPase activity, purified from porcine brain cortex, was investigated. Additionally, the complexes were tested on human lymphocytes, and incidence of micronuclei and cell proliferation index was determined. Concentration-dependent inhibition of the enzyme for all three compounds was obtained, but with differing potencies. Calculated IC_{50} from Hill analysis were (in M): 5.75×10^{-7}, 5.50×10^{-6} and 3.98×10^{-5}, for $H[AuCl_4]$, $[Au(DMSO)_2Cl_2]Cl$ and $[Au(bipy)Cl_2]Cl$, respectively, while Hill coefficient values, n, were above 1 in all cases. This inhibition can be prevented using –SH donating ligands such as L-Cys and glutathione, and these ligands can also cause a recovery of the enzyme activity after the induced inhibition. Kinetic analysis demonstrated that each of the studied gold(III) complexes affects Na^+/K^+ ATPase reducing maximum enzymatic velocity, V_{max}, but not significantly changing the affinity for the substrate (K_M value), implying a noncompetitive mode of the interaction. Furthermore, among investigated gold(III) complexes, the $[Au(bipy)Cl_2]Cl$ complex exhibits a strong cytotoxic effect on human lymphocytes, which suggests its potential for use in antitumor therapy.

1. Introduction

The interest in the chemistry of gold (Au) compounds has received much attention over the last two decades because of their traditional use in medicine for the treatment of various diseases [1,2]. Au(III) complexes are isoelectronic and isostructural with Pt(II) compounds, both metals have the same electronic configuration (d^8) and form square planar complexes. Moreover, Au(III) compounds generally exhibit interesting cytotoxic and antitumor properties and are in the focus of interest in the area of metal based antitumor agents, because of their potential application as alternative to platinum drugs [3–6]. However, Au(III) compounds were neglected for a long time because of their relative instability [6]. It was recently found that some simple mononuclear Au(III) complexes are sufficiently stable under the physiological environment and display relevant cell killing properties toward selected human tumor cell lines [7–9]. Since Pt(II) compounds express antitumor properties most probably due to the interaction with DNA [10], recent investigations pointed out that the interactions of cytotoxic Au(III) complexes with DNA are significantly different and weaker than those of platinum analogues [7,11]. Some gold compounds that were evaluated displayed biological effects that are mediated by an antimitochondrial mechanism rather than by direct DNA damage [8].

Substitution reactions of Au(III) complexes are faster than the d^8 Pt(II) square-planar complexes. Au(III) compounds easily reduce to Au(I) in physiological conditions due to their rapid kinetics and higher charge and redox potential [6,12,13]. It was demonstrated that sulfur-containing biomolecules have high affinity for platinum and gold complexes. While the reactions of some amino acids (L-Cys, L-methionine) and glutathione (GSH) with the Pt(II) and Pd(II) are a simple substitution process [14,15], the reaction of L-Cys and L-methionine with Au(III) complex was found to be initially fast, followed by the much slower process, which was attributed to reduction to Au(I) [16].

Na^+/K^+ ATPase (sodium potassium adenosinetriphosphatase, EC. 3.6.3.9) is membrane located enzyme found in all animal cells [17]. Its main function is to catalyze ATP hydrolysis. The energy liberated in this process is used as the driving force for active transport of monovalent cations (Na^+, K^+) through membranes [18]. The activity of this enzyme is very sensitive to the influence of various bioregulators, such as cardiac steroids, transition and heavy metals, as well as metal complexes [19–27]. Various metal ions binding to enzyme sulfhydryl groups have often been implicated in the inhibition of Na^+/K^+ ATPase activity [22,23,28]. Furthermore, nephrotoxicity, ototoxicity *etc.* of platinum anticancer drugs, such as cisplatin and chloroplatinic acid, are related to inhibition of Na^+/K^+ ATPase activity [24,29]. However, gold

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is one of the most potent nonspecific inhibitors of Na^+/K^+ ATPase, with characteristics differing from other metallic inhibitors of this enzyme system [30]. Besides, reduced metallic gold particles may cause cytotoxic effects, and also enhance Na^+/K^+ ATPase activity [31,32].

Recently, our work was concentrated on the *in vitro* investigations of the influence of some Pt(II) group complexes on Na^+/K^+ ATPase activity and the reactivation of the inhibited enzyme in the presence of GSH and L-Cys. Moreover, it was reported that GSH and L-Cys are potent enzyme activity reactivators, since they recover the metal ions that induced inhibition of some enzymes [14,33,34]. The present paper represents the continuation of our work concerning the influence of noble metal complexes on Na^+/K^+ ATPase activity. The interference of $[Au(DMSO)_2Cl_2]^+$ and $[Au(bipy)Cl_2]^+$ (bipy = 2,2′-bipyridine) complexes with Na^+/K^+ ATPase activity in the presence of L-Cys and GSH was studied and compared to the effect of $[AuCl_4^-]$ (Fig. 1). Besides, the study is broadened by the *in vitro* toxicity investigation of these complexes on human lymphocytes as a model system.

2. Experimental

2.1. Chemicals

All commercially available chemicals were of analytical grade. L-Cys (99.5%), GSH and $H[AuCl_4]$ were obtained from Fluka (Switzerland). $[Au(DMSO)_2Cl_2]Cl$ and $[Au(bipy)Cl_2]Cl$, were synthesized according to the published procedures [8,35,36]. The chemical analysis and UV–VIS spectral data were in good agreement with those obtained for the previous preparation. 1×10^{-3} M stock solutions of complexes in DMSO and 1×10^{-2} M ligands were prepared shortly before use. Working solutions were prepared by diluting the stock solutions to desired concentrations. Na^+/K^+ ATPase from porcine brain cortex (specific activity of 0.1 IU/mg solid) as well as some chemicals for medium assay (ATP, NaCl, KCl, $MgCl_2$, and Tris–HCl) were purchased from Sigma-Aldrich (Germany). Chemicals for determination of Na^+/K^+ ATPase activity (stannous chloride and ammonium molybdate) were from Merck (Germany). Deionized water was used throughout. PB-max karyotyping medium for cell culturing was obtained from Invitrogen-Gibco, Paisley, UK. For micronucleus assay, cytochalasin B and Giemsa stain were purchased from Sigma-Aldrich (Germany). Methanol and acetic acid were obtained from VWR (Germany).

2.2. Na^+/K^+ ATPase activity assay

Na^+/K^+ ATPase activity was determined in a standard incubation medium (200 μl), containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 2 mM ATP and protein (2 mg solid/ml) in the presence or absence (control) of the desired concentration of complex. Incubation mixtures were preincubated for 10 min at 37 °C in the presence or absence of inhibitor or water (control). The reaction was started by the addition of ATP, allowed to proceed for 15 min, and interrupted by the addition of the ice cold $HClO_4$ and immediate cooling on ice. The inorganic orthophosphate (Pi) liberated from the hydrolysis of ATP was measured using modified spectrophotometric procedure [37] based on the stannous chloride method, by reading the absorbance at 690 nm. The results are expressed as the mean percentage of enzyme activity relative to the corresponding control value. All experiments were performed in triplicate.

The effect of L-Cys and GSH on the prevention of the Au(III) induced inhibition was measured under the same conditions as described above, with the ligand added to the assay medium before the exposure to metal complex. The reactivating effect was measured by adding L-Cys and GSH after a 15 minute preincubation with the Au(III) complexes.

2.3. Lymphocyte cultures

Blood samples were obtained from three healthy, non-smoking young male volunteer donors in accordance with current Health and Ethical regulations in Serbia [38].

Aliquots of heparinized whole blood (0.5 ml) were placed in cultures containing PB-max karyotyping medium (Invitrogen-Gibco, Paisley, UK)

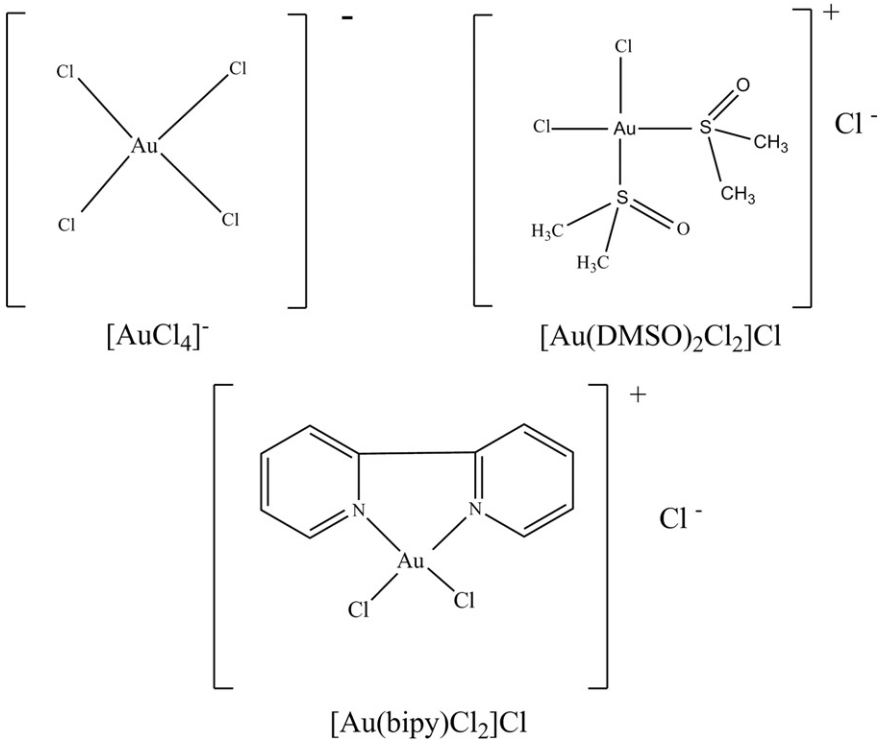

Fig. 1. Structural formulas of the investigated Au(III) complexes.

and treated with increasing concentration of gold complexes (final concentrations from 10^{-8} M to 10^{-4} M). Untreated cell cultures served as controls.

2.4. Micronucleus assay

For micronuclei preparation, the cytokinesis block method of Fenech [39] was used. Cytochalasin B at a final concentration of 4 μg/ml was added to each culture after 44 h of incubation in order to inhibit cytokinesis. The lymphocyte cultures were incubated further for 28 h. Cells were collected by centrifugation and treated with hypotonic solution at 37 °C. Hypotonic solution consisted of 0.56% KCl and 0.90% NaCl (mixed in equal volumes). Cell suspension was fixed in methanol/acetic acid (3:1), washed three times with fixative, and dropped onto a clean slide. Slides were air dried and stained in alkaline Giemsa (2%). For each sample, at least 1000 binucleated cells were scored and micronuclei were recorded using an AxioImager A1 microscope (Carl Zeiss, Jena, Germany) with 400× or 1000× magnification.

2.5. Cell proliferation index

A cytokinesis-block proliferation index (CBPI) was calculated according to method of Surrales at al. [40] as follows: CBPI = MI + 2MII + 3(MIII + MIV)/N, where MI–MIV represent the number of cells with one to four nuclei, respectively, and N is the number of cells scored.

2.6. Statistical analysis

Statistical analysis was performed using the statistical software package Statistica 8.0, and OriginPro 8 for Microsoft Windows. Statistical analysis was done using Student's *t* test and product–moment and partial correlations. P values less than 0.05 were considered significant and less than 0.001 were considered highly significant. Results are expressed as percentage of control.

2.7. Instrumentation

The absorption spectra were measured using the GBC Cintra 10 and Perkin Elmer Lambda 35 spectrophotometers with thermostated 1.00 cm quartz cell, in the wavelength range from 200 to 500 nm. The micronuclei were recorded using an AxioImager A1 microscope (Carl Zeiss, Germany) with 400× or 1000× magnification. pH values of the solutions were measured by a Methronom pH meter, Model 713.

3. Results

3.1. Effect of gold(III) complexes on Na^+/K^+ ATPase activity

The effect of H[$AuCl_4$], [$AuCl_2(DMSO)_2$]Cl and [$AuCl_2$bipy]Cl on the ATP hydrolysis catalyzed by Na^+/K^+ ATPase was investigated after addition of the complexes to the reaction mixture in the concentration range from 1×10^{-8} to 1×10^{-4} M. The results indicate that the complexes inhibit Na^+/K^+ ATPase activity in a concentration-dependent manner. The dependence of relative enzyme activity (REA), expressed as a percentage of the control value (Na^+/K^+ ATPase activity obtained without inhibitor), on the concentration of Au(III) complexes fits a sigmoid function (Eq. (1), Fig. 2).

$$y = \frac{A_1 - A_2}{1 + (x - x_0)^p} + A_2 \quad (1)$$

where x is inhibitor concentration in M, x_0 is equal to IC_{50} value (see below) and y is REA. A1, A2 and p are constant parameters ($A_1 \approx 100$, $A_2 \approx 0$, p is equal to Hill coefficient value, n (Table 1)).

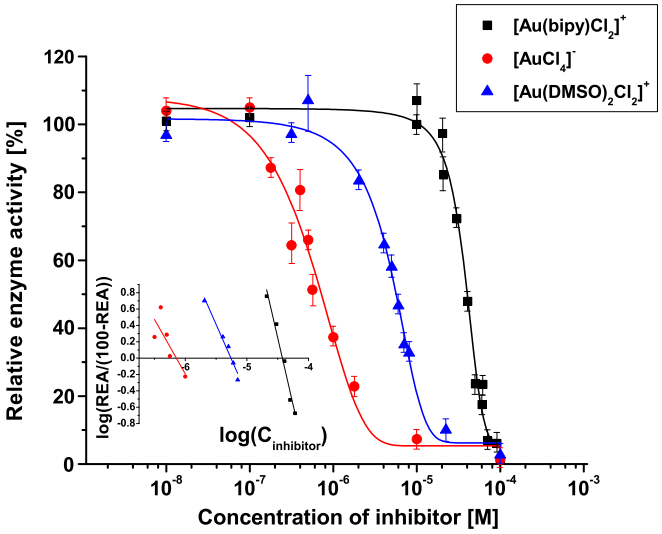

Fig. 2. Inhibiton of Na^+/K^+ ATPase with Au(III) complexes. Inset: Hill analysis of inhibition curves.

The concentration range of inhibitors relative to the linear region of the sigmoid plot was used for Hill analysis according to Eq. (2) (Fig. 2 (inset)).

$$\log\left(\frac{REA}{100 - REA}\right) = -n\log[I] + n\log IC_{50} \quad (2)$$

where, [I] is complex concentration, IC_{50} is half maximum inhibitory activity (*i.e.* the concentration of the complex with the ability to inhibit 50% of Na^+/K^+ ATPase activity after some exposure time) and n is Hill coefficient. The values of inhibition parameters (IC_{50} and n) determined by sigmoid fitting the experimental data and Hill analysis are given in Table 1. The obtained results suggest a significant difference in inhibitory potency of the investigated complexes toward Na^+/K^+ ATPase activity. Thus, H[$AuCl_4$] ($IC_{50} = (5.75 \pm 0.02) \times 10^{-7}$ M) exhibits ten times stronger inhibition than [$AuCl_2(DMSO)_2$]Cl ($IC_{50} = (5.50 \pm 0.02) \times 10^{-6}$ M), while Na^+/K^+ ATPase is a hundred times less sensitive toward [$AuCl_2$bipy]Cl ($IC_{50} = (3.98 \pm 0.02) \times 10^{-5}$ M) compared to H[$AuCl_4$]. Hill coefficient values (Table 1) above 1 ($n > 1$) indicate a positive cooperation for all complexes binding to Na^+/K^+ ATPase.

3.2. Prevention and recovery of gold(III) complexes induced Na^+/K^+ ATPase inhibition by L-Cys and gluthatione

Two series of experiments were performed to elucidate the effect of –SH containing ligands, L-Cys and GSH, on the inhibition of Na^+/K^+ ATPase activity. In the first experiment, the enzyme was preincubated with varying concentrations (from 1×10^{-6} to 1×10^{-3} M) of L-Cys or GSH for 15 min at 37 °C. Afterwards, the Au(III) complexes were added in the concentrations that cause 20% (IC_{20}), 50% (IC_{50}) and almost complete (IC_{100}) decreasing the Na^+/K^+ ATPase activity (see Fig. 2), and kept in contact with the enzyme and the ligand for 10 min. The dependence of the relative enzymatic activity *vs.* the concentration of

Table 1
IC_{50} and Hill coefficient (n) values for inhibition of Na^+/K^+ ATPase induced by Au(III) complexes.

Complex	Sigmoid fit	Hill analysis	
	IC_{50} [M]	IC_{50} [M]	n
$[AuCl_4]^-$	$(7.19 \pm 0.02) \times 10^{-7}$	$(7.24 \pm 0.02) \times 10^{-7}$	5.26 ± 0.01
$[Au(DMSO)_2Cl_2]^+$	$(5.50 \pm 0.02) \times 10^{-6}$	$(5.49 \pm 0.02) \times 10^{-7}$	2.25 ± 0.01
$[Au(bipy)Cl_2]^+$	$(3.88 \pm 0.02) \times 10^{-5}$	$(3.84 \pm 0.02) \times 10^{-6}$	4.52 ± 0.01

the –SH containing ligands in the presence of IC_{20}, IC_{50} and IC_{100} of complexes is presented in Fig. 3. The results show that there is a dose-dependent prevention of enzyme inhibition in the presence of L-Cys or GSH. It is obvious from Fig. 3 that the 1 mM ligand concentration results in the marked prevention of the enzyme inhibition. In the case of GSH, even 0.1 mM induces a significant increase in the remaining enzyme activity (about 40%) in the presence of IC_{100} complexes. This fact indicates a higher capability of this –SH containing ligand, compared with L-Cys, to prevent Na^+/K^+ ATPase inhibition by Au(III) complexes. Approximately complete prevention was only obtained by the action of 1 mM GSH or L-Cys at IC_{20} of each of three complexes.

The results obtained for the described prevention by the –SH containing ligands showed that it was reasonable to investigate the ability of L-Cys and GSH to recover Na^+/K^+ ATPase activity after the inhibition induced by the appropriate Au(III) complexes concentrations. In another series of experiments, L-Cys or GSH, in the concentration range from 1×10^{-6} to 1×10^{-3} M, was added into the incubation mixture 10 min after the enzyme had been exposed to IC_{20}, IC_{50} or IC_{100} of each inhibitor (at 37 °C). This preincubation period was long enough to establish the equilibrium between the complexes and enzyme. After 15 min contact between the ligand and formed enzyme–inhibitor complex, the enzyme reaction was started by ATP addition. The results presented in Fig. 4 display that the applied thiols possess a dose-dependent recovery effect on the diminished Na^+/K^+ ATPase activity exposed to the inhibiting

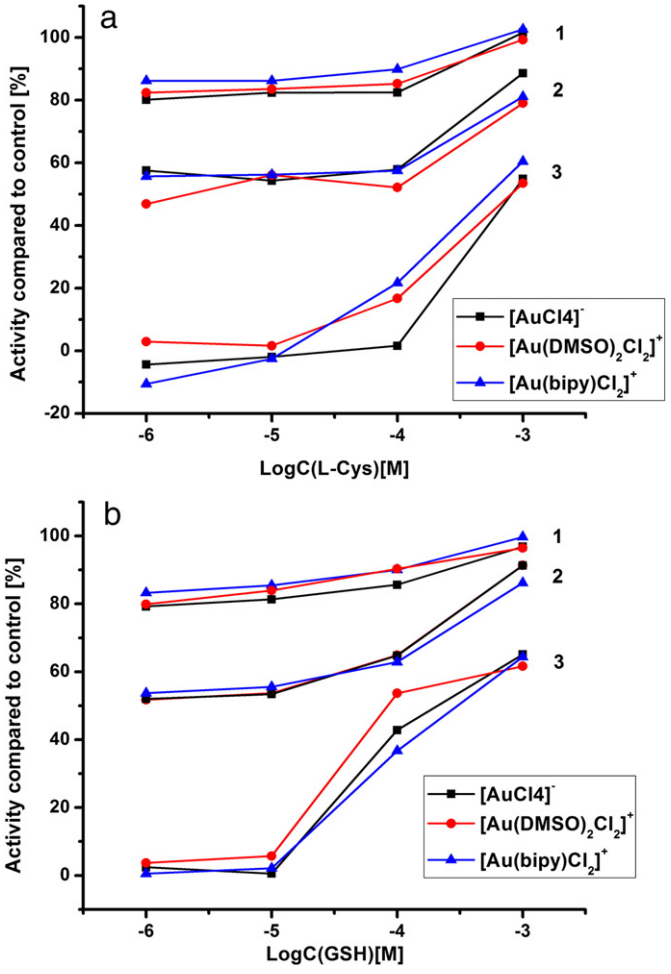

Fig. 3. Preventive effects of L-Cys (a) and GSH (b) on Na^+/K^+ ATPase activity in the presence of Au(III) complexes at concentrations which induce 20% (line group 1), 50% (line group 2) and 100% (line group 3) enzyme inhibition.

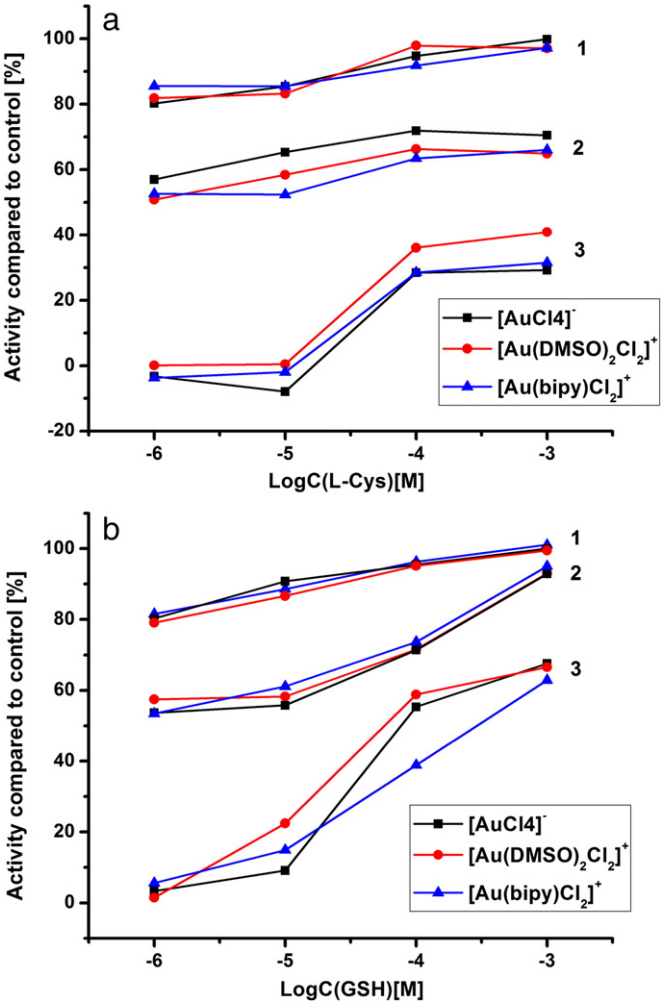

Fig. 4. Effects of L-Cys (a) and GSH (b) on the reactivation of the Na^+/K^+ ATPase activity inhibited in the presence of Au(III) complexes at concentrations which induce 20% (line group 1), 50% (line group 2) and 100% (line group 3) enzyme inhibition.

concentrations of complexes. Similar to the prevention of the inhibition, the ligand concentration of 1 mM leads to the noticeable regeneration of the decreased enzyme activity induced by the investigated Au(III) complexes (IC_{20}, IC_{50} and IC_{100}). Additionally, GSH demonstrates a stronger recovery capability than L-Cys, at higher concentrations, for IC_{50} and IC_{100} (Fig. 4, line groups 2 and 3). Also, 0.1 mM L-Cys addition, as well as GSH, significantly recovers the total enzyme inactivation caused by each of the inhibitors (Fig. 4, line group 3). The (approximately) complete regeneration is only possible at less enzyme inactivation (20% inhibited activity) using higher concentrations of either reactivators (Fig. 4, line group 1).

3.3. Kinetic analysis

The detailed kinetic analysis of Na^+/K^+ ATPase inhibition induced by the investigated complexes was performed in order to determine the mode of interaction between the enzyme and inhibitors. For this purpose, the initial reaction rate (v_o) in the presence and absence of Au(III) complexes was determined as the function of $MgATP^{2-}$ in the concentration range from 0.15 to 5 mM, while maintaining the concentrations of other substances in the reaction mixture constant. The inhibitor concentrations were selected from the inhibition curves (Fig. 2), as the concentrations that inhibited the enzyme activity were about 50%. These were 5×10^{-5} M for $[Au(bipy)Cl_2]^+$, 5×10^{-6} M

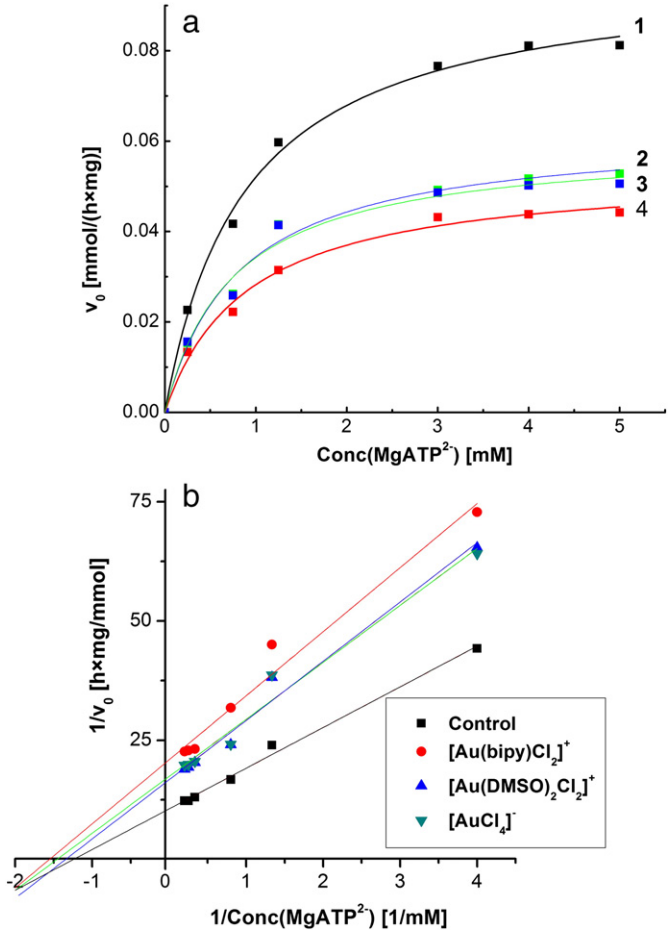

Fig. 5. a) Initial reaction rate (v_o) vs. $MgATP^{2-}$ concentration in the absence (1) and presence of 5×10^{-6} M for $[Au(DMSO)_2Cl_2]^+$ (2), 5×10^{-5} M $[Au(bipy)Cl_2]^+$ (3) and 5×10^{-7} M $[AuCl_4]^-$ (4). The values given are the mean of at least three experiments ± S.E.M. b) The Lineweaver–Burk transformation of the data.

for $[Au(DMSO)_2Cl_2]^+$ and 5×10^{-7} M for $[AuCl_4]^-$. The obtained results fit the typical hyperbolic Michaelis–Menten curve (Fig. 5a).

Kinetic parameters, K_M and V_{max}, were calculated from hyperbola function (Fig. 5a), as well as using linear Lineweaver–Burk transformation. The dependences of $1/v_o$ *vs.* $1/C_{MgATP}^{2-}$ were presented and linear plots were obtained in all cases (Fig. 5b). The values of K_M and V_{max} were evaluated from the intercept of the linear plots with x and y axes, ($1/K_M$ and $1/V_{max}$, respectively). The results are summarized in Table 2. V_{max} decreased in the presence of the inhibitors in all cases, while K_M remained constant, compared to the control sample without the inhibitor (Table 2). The obtained values suggest the noncompetitive reversible type of Na^+/K^+ ATPase inhibition by the used Au(III) complexes, since the K_M value was not affected by the inhibitor binding, while V_{max} decreased in the presence of each of the complexes.

Table 2
The values of K_M and V_{max} determined from Michaelis–Menten hyperbola function and its linear Lineweaver–Burk transformation.

Complex	K_M [mM]	V_{max} [mmol/h/mg]
Control	0.79 ± 0.07	0.094 ± 0.005
$[AuCl_4]^-$	0.63 ± 0.10	0.047 ± 0.004
$[Au(DMSO)_2Cl_2]^+$	0.73 ± 0.11	0.059 ± 0.005
$[Au(bipy)Cl_2]^+$	0.67 ± 0.11	0.057 ± 0.005

3.4. Influence of gold(III) complexes on human lymphocytes

The effects of increasing concentrations of Au(III) complexes on the incidence of micronuclei and proliferation index in lymphocyte cultures are presented in Fig. 6.

In lymphocyte cultures, significant concentration-dependent increase ($p < 0.05$) of the incidence of micronuclei was observed in all samples treated with $[Au(bipy)Cl_2]^+$. At concentration range from 10^{-8} M–10^{-5} M, the incidence of micronuclei was enhanced by 10.41%–30.72% relative to control, while at the highest employed concentration (10^{-4} M) the 4-fold increase of the incidence of micronuclei was observed ($p < 0.001$). In lymphocyte cultures treated with $[AuCl_4]^-$ micronuclei formation slightly increased in a dose-dependent manner up to concentration of 10^{-7} M, afterwards it significantly ($p < 0.05$) decreased compared to control (by 34.47%–49.91%). Similar reduction in micronuclei formation (by approximately 50%, $p < 0.001$) was observed in all samples treated with increasing concentrations of $[Au(DMSO)_2Cl_2]^+$.

Concentration-dependent, yet insignificant increase of CBPI was observed in samples treated with all the investigated Au complexes. However, at the highest employed concentration (10^{-4} M) of complex $[Au(bipy)Cl_2]^+$, the significant suppression of cell proliferation was observed ($p < 0.05$). The incidence of micronuclei and cytokinesis-block proliferation index correlated inversely, and was statistically significant ($r = -0.98$, $p < 0.05$). In samples treated with $[Au(DMSO)_2Cl_2]^+$ and $[AuCl_4]^-$, no significant correlation between micronuclei induction and cell proliferation index was observed.

4. Discussion

In this study we explored the influence of three selected Au(III) complexes on the activity of commercially available porcine cerebral cortex Na^+/K^+ ATPase. The obtained results (Fig. 2) demonstrate a concentration-dependent inhibitory effect of all the explored compounds on the enzyme activity, but with variable potencies. These results are in agreement with previous studies that have already demonstrated the affinity of gold and similar transition and heavy metals (palladium, platinum *etc.*) [33,34,41] to react with –SH groups of proteins. For all studied Au(III) complexes the action of gold is more complex than simple interaction with the –SH groups, and the redox reactions with L-Cys residues and disulfide bonds of Na^+/K^+ ATPase must be also taken into account. The disulfide bridges in the Na^+/K^+ ATPase are required for enzyme functionality. The removal of disulfide bonds from the ß subunit due to the redox reactions of gold complexes can lead to the significant functional alterations [42]. Moreover, the reduction of complexes with the tendency $[AuCl_4]^- > [Au(DMSO)_2Cl_2]^+ > [Au(bipy)_2Cl_2]^+$ can occur and the two oxidation states of gold may behave differentially toward the enzyme [43]. It is interesting to note here that a gradient in the tendency towards reduction correlates with the potency of inhibitors, but we believe that it is only one of the contributing factors in the process. Stability of the complex and size of the ligand also affect the potency of an inhibitor. In all cases the high Hill coefficient is observed. This suggests a strong positive cooperation of the inhibitor binding to the enzyme. In essence, after the first molecule of the inhibitor has reacted with the enzyme, other molecules bind even easier.

The prevention of inhibition and reactivation of the inhibited enzyme have been achieved using potent –SH donors. The likely reason for prevention of inhibition is the fast formation of the inactive $[AuCl(L\text{-}Cys)(DMSO)_2]^+$ complex analogous to the $[PtCl(L\text{-}Cys)(DMSO)_2]$ of Bugarčić et al. [44], prior to the formation of the [Au(III)complex (enzyme)]$^+$ complex. Further reduction of Au(III), resulting in the formation of Au(I) and Au colloids in the slower second reaction step, is likely in the prevention and the recovery experiments when GSH and L-Cys were added to the media. This process is common for –SH

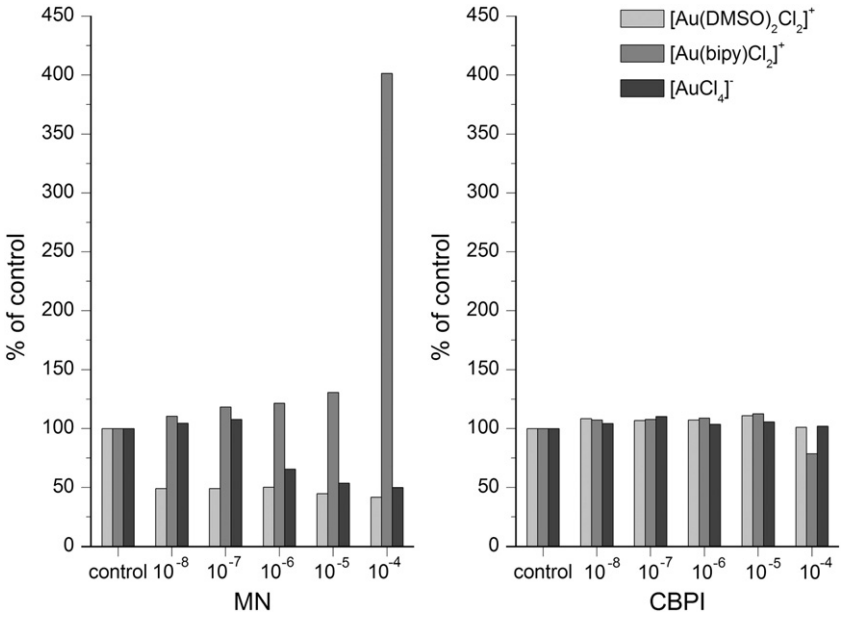

Fig. 6. Incidence of micronuclei (MN) and cytochalasine B proliferation index (CBPI) (expressed as percentage of control) in lymphocyte cultures treated with increasing concentrations of Au(III) complexes.

containing ligands, and the fast replacement of the ligand in coordination sphere of Au(III) complexes leads usually to reduction [16]. This reduction leads also to the formation of colloidal Au particles which are even able to enhance the enzyme activity [31]. The recovery of the activity was achieved when the concentration of thiols was equal or higher than the concentration of the complexes. These results can be explained by enzyme extruding from [Au(III)complex(enzyme)] complex and its substitution with smaller and more reactive –SH donor. A similar effect was earlier demonstrated on Pd(II) complexes [34]. In addition, Au(III) compounds can oxidatively cleave disulfide bonds of β unit, and this may contribute to the lost non-recoverable enzyme activity [42,45,46].

Kinetic studies have shown that the inhibition is a noncompetitive type, and that K_M remains constant after the inhibition. This gives some insights on the mechanism of inhibition, suggesting that it likely involves binding to the –SH groups that are not in the ATP binding site of the enzyme. The investigated Na^+/K^+ ATPase in these experiments is a membrane enzyme, purified from porcine cerebral cortex. In such prepared plasma membrane fragments, these –SH groups are probably not encapsulated, and they are accessible to the Au(III) compounds. For that reason, the obtained *in vitro* results would be different from the potential interactions of the Au(III) compounds and Na^+/K^+ ATPase using more complex model systems such as synaptosomes, cell cultures, experimental animals. Since the enzyme has around 64 different –SH groups, it would be difficult to determine the precise amino acid residues that are at the inhibitor binding sites. These results are in agreement with previous studies [34].

Biological investigations were performed on human lymphocytes *in vitro* employing cytokinesis-block micronucleus assay designed by Fenech [39]. The test measures DNA damage, cytostasis and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated (BN) cells who display small round bodies-micronuclei (MN). Micronuclei arise from acentric chromosome structures (misrepaired DNA damages) or whole chromosome which was not carried to the opposite poles during the anaphase. Their formation results in the daughter cell lacking a part or all of a chromosome. Incidence of micronuclei serves as an index of genetic damage. Cytostatic effects are measured by proportion of mono–bi- and multinucleated cells-cytochalasin B proliferation index (CBPI). These parameters correspond to the amount of DNA damage in a living cell and to the cytotoxicity of the tested complex, respectively. Except for a modest increase of proliferative capacity of cells, which might suggest disturbed function of cell cycle checkpoints, the complexes $H[AuCl_4]$ and $[Au(DMSO)_2Cl_2]Cl$ did not induce cytotoxic effects in human lymphocytes. However, extensive and dose-dependent damage to DNA (observed as a rise in the incidence of micronuclei) was observed after treatment of the cells with the complex $[Au(bipy)Cl_2]Cl$, which caused cytotoxic effects as revealed by significant enhancement of micronuclei incidence and suppression of cell proliferation, particularly at the highest concentration employed. These results are in accordance with several studies that reported antiproliferative properties of Au(III) complexes [8,11,12]. Since current cancer therapy mostly relies on DNA damaging agents to induce antiproliferative effects in cancer cells, these results suggest the potential use of $[Au(bipy)Cl_2]Cl$ in antitumor therapy. However, potential occurrence of severe side-effects in cancer therapy using Au or other metal complexes should not be underestimated. Taken together, results of this study clearly indicate that the micronuclei induction and proliferative potential of lymphocytes strongly depend not only on concentration but also on the type of the complexes.

5. Conclusion

We conclude that the complexes we explored have shown a dose-dependent inhibition of the porcine brain cortex Na^+/K^+ ATPase. This inhibition is noncompetitive and it is achieved *via* the –SH groups of the enzyme. This inhibition can be prevented if –SH containing ligands such as L-Cys and glutathione are present in the reaction medium, and it can be partially reversed if the inhibited enzyme is treated with those same –SH containing ligands. The biological effects upon the living cells match those that are seen with other similar inhibitors — the cell's normal life cycle and DNA repair process is disrupted.

In summary, elucidation of the reaction parameters between Au(III) complexes and biomolecules is helpful in: a) estimation of potential toxicity of complexes that might be further developed and used in the tumor therapy and, thus for b) development of

approaches for prevention of side effects of the therapy. This prevention includes the administration of –SH containing molecules.

Abbreviations

bipy	2,2′-bipyridine
CBPI	cytochalasin B proliferation index
GSH	glutathione
MN	micronuclei
REA	relative enzyme activity

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INTERACTION OF GOLD NANOPARTICLES WITH RAT BRAIN SYNAPTOSOMAL PLASMA MEMBRANE Na^+/K^+- ATPase AND Mg^{2+}-ATPase

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The aim of the work was to investigate the interaction between borate capped gold nanoparticles (NPs) and the rat brain synaptosomal plasma membranes (SPM), as well as the effects of these NPs on SPM Na^+/K^+- ATPase and Mg^{2+}-ATPase activity. The changes in the UV-vis spectra of NPs and SPM assembly suggested the agglomeration and precipitation of NPs. FTIR measurements indicated that both protein –SH and $–NH_2$ groups and positively charged membrane fragments were implicated in the adhesion of SPM to the surface of NPs. AFM showed an increase in the particularization of the SPM material after mixing with gold NPs. Influence of gold NPs on Na^+/K^+-ATPase and Mg^{2+}-ATPase activity was investigated as the function of NPs and protein concentration, preincubation time and also in the presence of various concentrations of ouabain, the specific enzyme inhibitor. NPs induced the stimulation of Na^+/K^+-ATPase activity for more than 100%, since Mg^{2+}-ATPase activity reminded unaffected. We propose that this stimulation of enzyme activity was a consequence of an increase of the active surface of membranes.

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Keywords: Gold nanoparticles, Na^+/K^+-ATPase, Mg^{2+}-ATPase, synaptosomal plasma membranes, biofunctionalization.

1. Introduction

Gold nanoparticles (Au NPs) are a very attractive tool in biomedical research, because they have repeatedly shown great potential as substance carriers, active surfaces and even biologically active agents [1]. Since they can be readily taken up by cells [2], they are proposed in the medical sector as new tools in diagnostics [3] and drug delivery systems [4]. Although GNPs are considered being inert in biomedical applications [5], the size dependent cytotoxicity towards different cell types, with smaller particles being more toxic has been reported [6, 7].

The particles themselves are easily modified due to the gold's ability to bond with biologically important molecular groups such as amines, thiols, and carboxyl groups from amino acids and proteins [8, 9]. They have also been used as immobilization matrices for enzymes, where the immobilized enzyme was found to be more stable compared to free enzyme [10, 11]. Of all available molecular targets in living tissue, proteins have shown the greatest affinity towards GNPs. The conjugation of proteins with GNPs stabilized the system and also introduced biocompatibile functionalities into the nanoparticles for further biological application. [12-14] The binding of the proteins to nanoparticles might occur by electrostatic forces between the surface-terminated negatively charged groups capping the nanoparticle and the positively charged functional groups of the protein (e.g. aminoacids residues) [9, 15, 16].

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Na^+/K^+-ATPase and Mg^{2+}-ATPase (ecto-ATPase) are membrane enzymes ubiquitous in animal cells that involve 5'-adenosine triphosphate (ATP) as a substrate for their functioning. Na^+/K^+-ATPase plays a key role in the active transport of monovalent cations (Na^+ and K^+) across the membrane [17]. Beside its transporter function, Na^+/K^+-ATPase acts as the receptor for cardiac glycosides such as ouabain like compounds [18], which are the specific inhibitors of the enzyme. The enzyme is composed of α-subunit, which contains the adenosinetriphosphate (ATP), Na^+, K^+ and ouabain binding sites, as well as the site for phosphorylation and β-subunit, which stabilizes the K^+ binding cage. The ouabain insensitive ecto-adenosine triphosphatase (Mg^{2+}-ATPase) represents an integral membrane protein that, in the presence of divalent cations (Ca^{2+} or Mg^{2+}), hydrolyses extracellular nucleotides because of the outward orientation of its active site [19]. It is much less well characterized than sodium pump, but apparently consists of at least two forms with different molecular weights and sensitivity to metal ions. There is a great number of organic molecules and metallic ions that can modulate the activity of these enzymes [20, 21]. Literature surveys suggest that some NPs (Cu, TiO_2) inhibit Na^+/K^+ ATPase activity and induce oxidative stress [22, 23]. Also, it was shown that some of the gold compounds have an inhibitory effect upon enzyme [24]. On the contrary, the literature data indicate that gold and silver nanoparticles stimulated ATPases activity of native and rehydrated cells of *Escherichia coli* [25]. In general, there are some fundamental differences between the physiological effects of metal ions and NPs. The chemistry and behavior of metal nanoparticles involve dynamic aspects of aggregation theory, rather than equilibrium models traditionally used for free metal ions.

One of the available model systems for the study of Na^+/K^+-ATPase and Mg^{2+}-ATPase are the rat brain synaptosomal plasma membrane fragments (SPM) [26]. In this paper we describe the influence of borate capped Au NPs of various sizes on SPM Na^+/K^+-ATPase and Mg^{2+}-ATPase activity in this model system. In addition, the interaction of SPM with Au NPs was characterized by application of various techniques, such as UV-Vis spectrophotometry, Fourier transformation infrared spectroscopy (FTIR) and atomic force microscopy (AFM).

2. Experimental

2.1 Materials

Gold (III) chloride trihydrate ($HAuCl_4 \times 3H_2O$), sodium borohydride ($NaBH_4$, 99%), perchloric acid ($HClO_4$), ouabain, all from Aldrich, were used as received. Rat SPM were isolated according to a previously described method and stored at -80° C until use [26]. Adenosinetriphosphate (ATP), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), tris(hydroxymethyl) aminomethane (TrisHCl), were purchased from Sigma Aldrich (Germany). Stannous chloride ($SnCl_2$) and ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}$) were from Merck (Darmstadt, Germany). Water purified with a Millipore Milli-Q water system was used for preparing all solutions.

2.2 Preparation of gold nanoparticles

Au NPs were obtained by the reduction of $HAuCl_4$ with $NaBH_4$. Precisely, 100 mL of 0.2 mM $HAuCl_4$ was reduced by 5.3 mM of $NaBH_4$ at room temperature, to yield a ruby-red solution. The calculated value of NPs concentration was 2.8×10^{-9} M, assuming that the reduction from gold(III) to gold atoms was 100% complete.

2.3 ATPase activity measurements

The incubation mixture contained 400 mM NaCl; 80 mM KCl; 20 mM $MgCl_2$, 200 mM Tris HCl, pH=7.4 and 25μL of SPM containing 1 mg/mL protein in total volume 200 μL at 37°C. 20 μL of 20 mM ATP was added to initiate the enzymatic reaction which was allowed to proceed for 15 min. Reaction was stopped by an addition of 22 μL of 3 M $HClO_4$ and by cooling of samples on ice. The concentration of liberated inorganic orthophosphate was determined

spectrophotometrically [26]. The activity obtained without NaCl and KCl in the medium assay was attributed to Mg^{2+}-ATPase. Na^+/K^+-ATPase activity was calculated as a difference between the total ATPase and Mg^{2+}-ATPase activity. The results represent the mean values of at least two experiments done in duplicate.

2.4 Apparatus

Transmission electron microscope (TEM, JOEL 100CX) was used to observe the morphology and average size of Au nanoparticles.

All spectrophotometric measurements were performed on a Perkin Elmer Lambda 35 UV-vis spectrophotometer with 1 cm path length quartz cuvette.

FTIR spectra were recorded on Thermo Electron Corporation Nicolet 380 FTIR Spectrophotometer with ATR (Attenuated Total Reflection) accessory, equipped with a diamond tip, in the region between 4000 and 500 cm^{-1}.

AFM measurements were carried out using commercial AFM VECCO Quadrexed Multi Mmode IIIE equipment operating in tapping mode (TMAFM) on highly oriented pyrolytic graphite (HOPG). Surface topography and phase images were simultaneously aquired using a commercial NanoScience-Team Nanotec GmbH SCN (Solid Nitride Cone) AFM probe, with tip radius lower than 10 nm. AFM observations were repeated on different areas from $2x2\mu m^2$ to $250x250nm^2$ on the same substrate at ambient laboratory conditions (about 20^oC)

Droplet-evaporation method was used for preparing AFM samples for analysis. A droplet of $2x10^{-9}$ M Au colloid or SPM containing 1 mg/mL of protein was deposited on freshly cleaned HOPG 1x1 cm. The droplet was then carefully washed after allowing the sample to sit about 15 minutes. For gold nanoparticles functionalized with protein, 100 µL of Au colloid was mixed with 25 µL of SPM. 15 min after mixing the sample was put on HOPG as described above.

3. Results

3.1 Spectrophotometric characterization of Au NPs interaction with SPM

The Au colloidal dispersion was used to investigate the interaction with SPM. According to TEM measurements (data not shown), the nanoparticles were spherical in shape with the average particle diameter 9.5±0.8 nm.

UV-vis spectroscopy was initially used to monitor spectral changes in colloidal solutions upon addition of SPM. The spectra were recorded before and after the mixing of 100 µl of SPM and 25µl of colloid solution containing $2x10^{-9}$ M NPs, under stirring at room temperature. This was then diluted with water to 1.5 mL and measured. The gold NPs show an intense surface plasmon resonance (SPR) band at 518 nm due to the collective excitation of conducting electrons in a metal (Fig. 1 curve 2).

Adding SPM into the gold colloidal solution, some spectral changes were observed. (Fig. 1 curves 3-5). The spectral changes of SPR band resulted from the replacement of borate ions on the NPs surface by the SPM molecules that caused a significant decrease in the stability ratio and aggregation of NPs. The agglomeration was extremely fast and fine precipitates were formed after 10 min of mixing with SPM. As a consequence of agglomeration, the corresponding SPR band was broadened and shifted to longer wavelengths due to dipole-dipole interactions of high induced dipole moment of NPs in aggregates. The SPR band completely disappeared after 20 min.

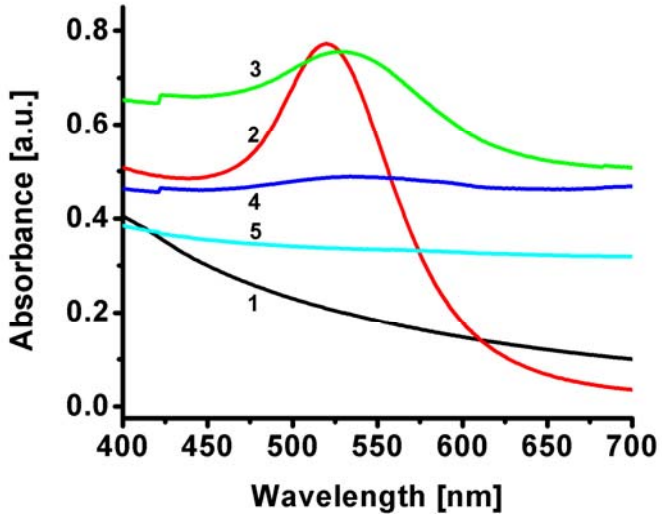

Fig. 1. UV-vis spectra of the solutions containing 66.6 µg/mL SPM (1) and 3.3x10^{-11} M Au NPs (2) and 1 min (3), 10 min (4) and 20 min (5) after the mixing.

3.2 FTIR characterization of Au NPs with SPM

FTIR spectra were recorded in order to evaluate the possible types of interactions that exist between the Au NPs and SPM. A number of vibration bands can be seen in the two different regions of the spectra as shown in Fig. 2. The FTIR spectrum of the SPM in water solution exhibits small bands located at 2940-2820 cm^{-1} related to the protein and lipid C-H asymmetric and symmetric stretching vibrations. The interaction Au NPs with SPM molecules leads to increase intensity and narrowing of the band at 2928 cm^{-1} due to the asymmetric stretching mode of the methylene groups in lipid sequences. Also, the rigid chain conformation on the NPs surface restricts protein mobility, resulting in the narrowing of the stretching bands [27, 28].

The multiply bonded CO group provides the intense bands between 2100 and 1900 cm^{-1}, clearly visible in the Fig.2. The new mode due to scissoring of a methylene group adjacent to the Au-S bond (δ_S) appears at 1430 cm^{-1} together with the weak bands around 1320 cm^{-1}. These bands are related to C-N, C-O vibrations of the protein backbone and amino acid residues and are shown in the spectrum. In addition, the weak band at 1050 cm^{-1} can be attributed to vibrations involving interaction between C-O stretching and C-N stretching. This band is shifted to 1160 cm^{-1}. Such effects are not surprising given the proximity of the gold surface to the bonds involved in these motions.

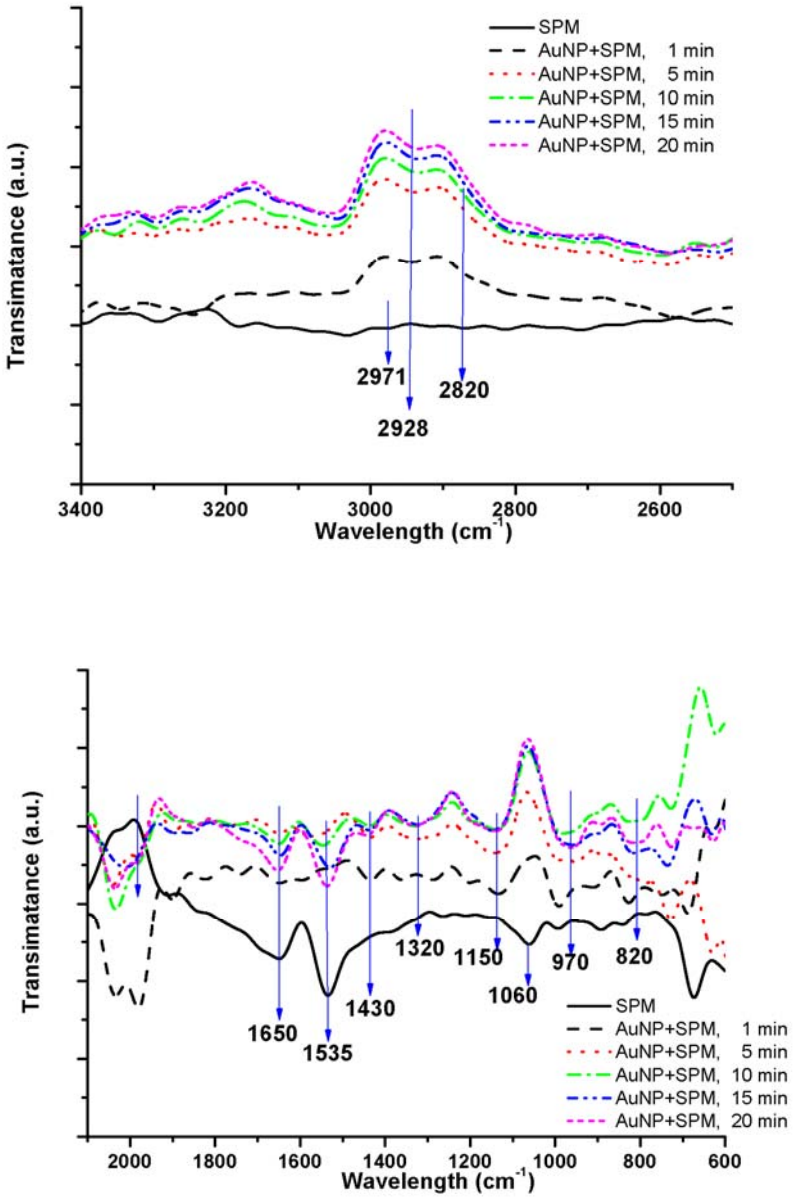

Fig. 2. FTIR spectra of SPM over time upon the addition of Au NPs

The specific region at 600-700 cm^{-1} assigned to the νS-C symmetric stretching vibration shows significant changes together with the bands at 970 and 820 cm^{-1} assigned to νAu-S symmetric stretching vibration, indicating that Au NPs and SPM interact via sulfur group [29]. The next characteristic vibration bands arise from the amide groups of proteins and provide information on its secondary structure, have been identified. Among them, amide I band (C=O stretching mode) appears at 1650 cm^{-1} and the amide II band due to the coupling C-N stretch to N-H bending at 1535 cm^{-1} [30]. The NPs interactions with SPM lead to increase intensity of these bands due to the interaction (via H-bonding) with protein C=O and C-N groups. Also, the slight displacement of the maximum amide I band from 1650 to 1652 cm^{-1} and amide II band from 1535 to 1537 cm^{-1} were observed upon addition of Au NPs.

3.3 AFM visualization of Au NPs interaction with SPM

The AFM measurements were performed with Au NPs in the absence and presence of SPM on HOPG. A representative AFM image of Au NPs in the absence of SPM is shown in Fig. 3. The section analysis of the image indicates the peaks, which correspond to the Au NPs anchored on the HOPG. The sizes of the particles were measured directly from AFM images. Single colloids display homogenous lateral and vertical dimensions. Height evolution was obtained by cross section profile in Fig. 3b. The size of nanoparticles (average diameter 9.4 nm) is in good agreement with the results obtained by TEM measurements.

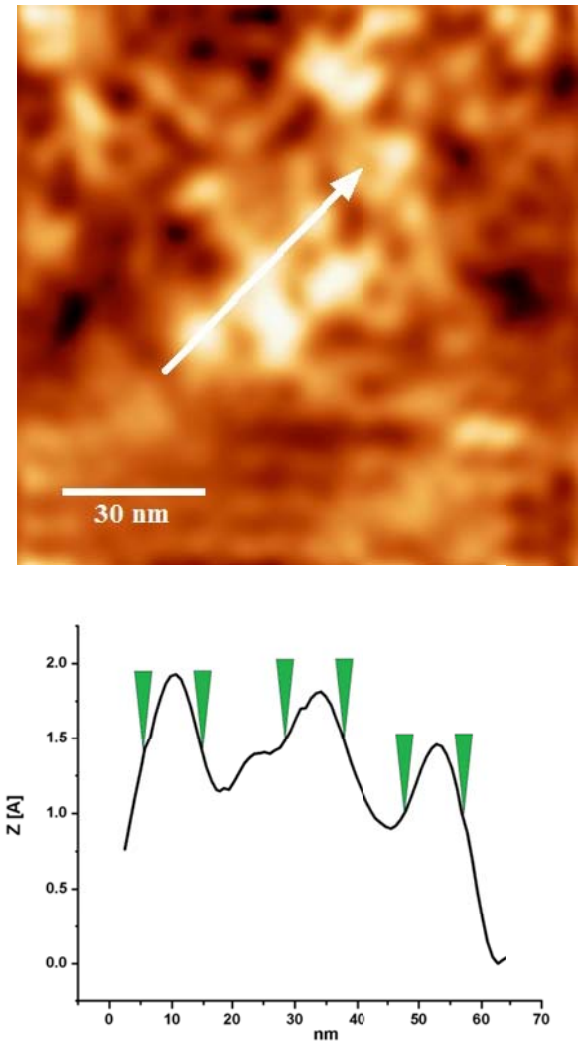

Fig. 3. AFM images of Au NPs deposited on HOPG after 15 min deposition time, scanned area 250x250 nm^2; a) 2D topography with cross section, b) cross section profile.

Fig. 4. shows the typical image of SPM membrane patches well distributed on the surface, in which the particles that are supposed to be membrane proteins are clearly observed. The stringy structure of SPM can be noticed.

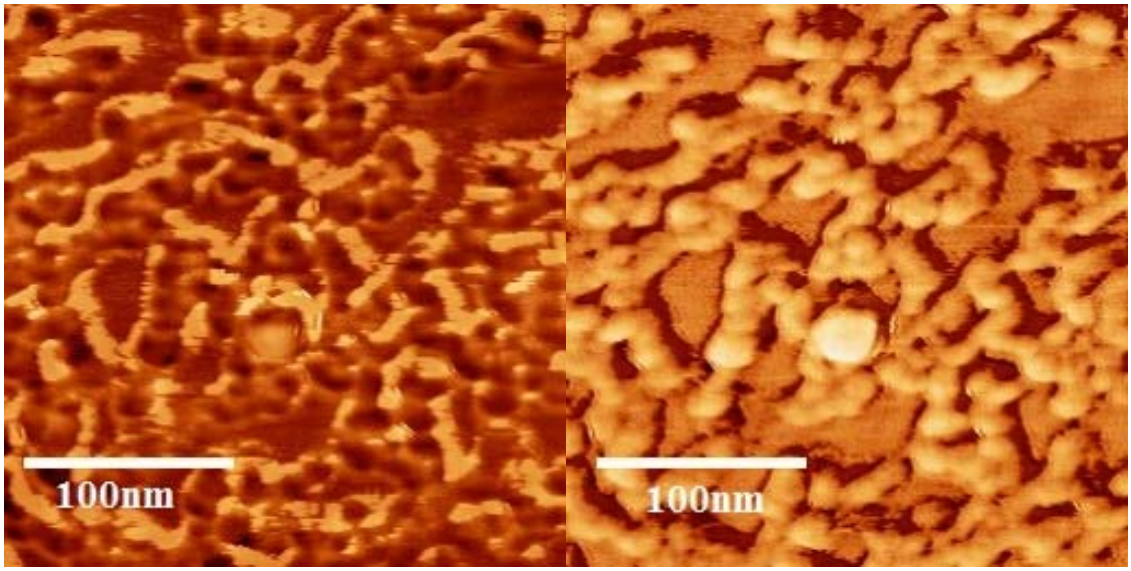

Fig. 4. AFM images of SPM deposited on HOPG after 15 min deposition time, scanned area 250x250 nm^2; a) - 2D topography, b) - 2D phase view.

Fig. 5. shows the mixture of SPM and Au NPs. It can be seen that there are no string-like structures and that most of the material is divided into smaller spherical aggregations. The results presented in Figs. 4 and 5 obtained from the AFM measurements indicated a change in the fine structure of SPM aggregates. A greater degree of particularization can be seen in SPM samples treated with colloid particles.

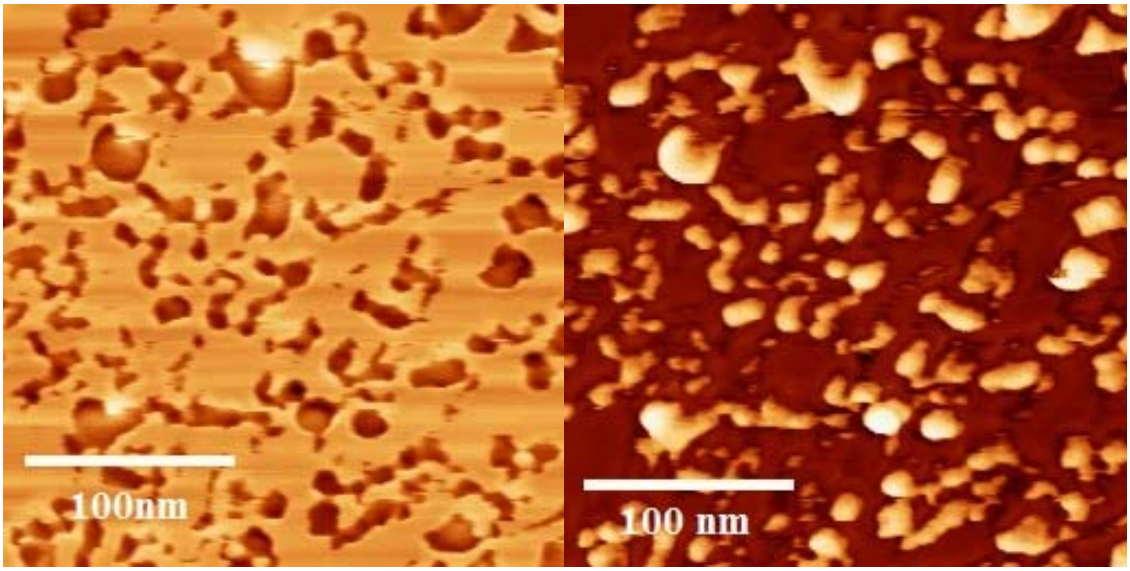

Fig. 5. AFM images of SPM treated with Au NPs deposited on HOPG after 15 min deposition time, scanned area 250x250 nm^2; a) - 2D topography, b) - 2D phase view.

3.4 Influence of Au NPs on ATPase activity

The influence of Au NPs within the concentration range from 10^{-10} – $5.25x10^{-10}$ M on the ATP hydrolysis catalyzed by SPM Na^+/K^+-ATPase and Mg^{2+}-ATPase was investigated, with the aim to find out if proteins retain their functionality in the presence of colloids. In this experiment the medium assay contained 20 μg protein. The catalytic reaction was started by addition of ATP

1 min after the nanoparticles were added into the medium assay and the results are presented in Fig.6.

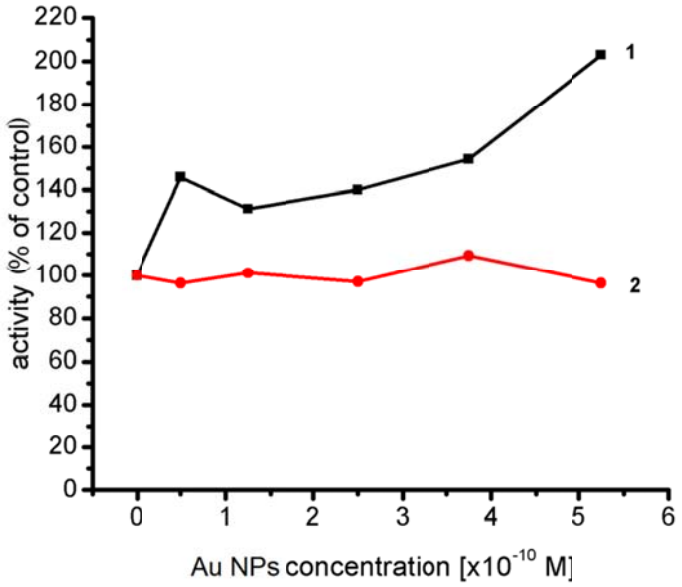

Fig. 6. Influence of Au NPs on Na^+/K^+-ATPase (1) and Mg^{2+}-ATPase (2) activity after 1 min preincubation.

The results indicated, that the borate capped Au NPs increased the activity of Na^+/K^+ -ATPase in the concentration dependent manner, compared to the control value without nanoparticles, since the activity of Mg^{2+}-ATPase remained constant. Moreover, the increse of the Na^+/K^+ -ATPase activity was more than 100% in the presence of $5.25x10^{-10}$ M Au NPs.

In the additional experiment the incubation time of SPM with nanoparticles (concentration was $5.25x10^{-10}$ M) was varied from 0.5 min to 120 min. The Na^+/K^+-ATPase activity increased for about the same value (100%) in all cases compared with the control value without nanoparticles, since Mg^{2+}-ATPase activity reminded unchanged.

Furthermore, an experiment with a constant concentration of nanoparticles (5.3×10^{-10}M) and a varying the amount of protein (from 5 μg – 40 μg total amount in medium assay) was carried out in order to investigate the trend of activity rise that was shown for Au NPs. The increase of activity for 85 – 110% compared to the control probe containing the same protein concentration without Au NPs was obtained in all cases.

In order to see if the interaction between Au NPs (concentration $5.25x10^{-10}$ M) and SPM Na^+/K^+-ATPase altered the enzyme selectivity towards its specific inhibitor, a set of activity measurements was carried out in the presence of ouabain, a selective inhibitor of Na^+/K^+-ATPase. Concentration span of ouabain in the assay was from $1x10^{-8}$ M to 10^{-5} M. Simultaneously, the inhibition was investigated in the medium assay of the same composition without Au NPs. Results show that the response of the enzymatic activity to the ouabain concentration is biphasic, indicating high and low affinity Na^+/K^+-ATPase isoforms (Fig.7). It is clear from the experimental results that the activity vs. ouabain concentration plots in both cases can be represented by the sum of two overlapping sigmoid curves, separated by a plateau as found in our previous work. [26, 31], [32]

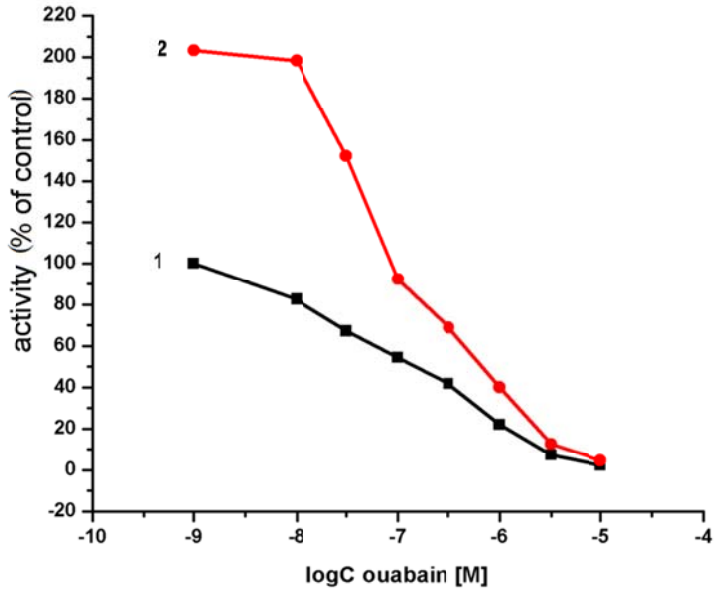

Fig 7. Inhibition of Na^+/K^+-ATPase activity by ouabain in the presence (1) and absence (2) of $5.25x10^{-10}$ M Au NPs.

The concentration dependent inhibition curves which correspond to high and low affinity inhibitor sites were obtained can be presented as the superposition of two sigmoid curves. Our results show that there was no major change in the IC_{50} values of ouabain. IC_{50} was found to be $(5.29\pm0.29)\cdot10^{-7}$ M for high and $(2.36\pm0.29)\cdot10^{-6}$ M for low affinity binding sites in both cases.

4. Discussion

The position of SPR band of Au NPs is affected by the particle size, shape, inter-particle distance and local dielectric environment of the particles [33]. Since the prepared Au NPs possessed a negative charge due to the adsorbed borate ions, the repulsive forces worked along particles and prevented their aggregation. Previous studies on nanoparticle-protein interactions suggest that adsorption of protein on the gold surface can induce formation of aggregates as a consequence of interaction between the positive surface residue of protein and the negative charge surface layer so the protein molecules constitute bridge among Au NPs [12, 34]. Also, during the formation of an adsorption layer on the Au surface, the anions can be replaced by protein upon adsorption, with the amino acids functional groups (amine and thiol groups) that interact directly with the Au surface [35-38]. Furthermore, the hydrophobic interactions among protein molecules within the protein layer adsorbed on the gold surface cannot be excluded. In the case of Au NPs, where borate anions could be easily replaced by the SPM molecules, a direct binding to the particle surface occurs *via* thiolate (–SH group) linkages through the cysteine residues [39], rather then $–NH_2$, which is consistent with the greater affinity of Au for –SH compared to $–NH_2$.

Consequently, these interactions can induce changes in the protein structure like a conformation changes, unfolds in protein near the particles surface, etc. The positions of amide I and amide II bands in the FTIR spectra of proteins are sensitive indicator of conformational changes in the protein secondary structure [30]. The amide I band is the sum of overlapping component bands (α-helix, β-sheet, β-turn and randomly coiled conformation), influenced by their different environment [40], while the amide II band indicates the amount of protein adsorbed on surface. The NPs interactions with SPM lead to the slight displacement and increase intensity of these bands. A probable reason for these changes is the orientation effects of the SPM molecule

during the interaction with gold surface. Nonetheless, it is worth noticing that no major rearrangement of the protein secondary structure upon interaction with gold surface occured.

AFM measurements enabled us to get information on the topography of membrane-bound proteins with extramembraneous protrusions. The lower domains can be described as the double lipid layer, since the higher domain represents the membrane parts with the high concentration of the transport protein [41],[42]. The results obtained by AFM measurements could imply that the presence of colloid particles caused further membrane fragmentation on a nano scale, resulting in greater effective membrane surface which in turn enables more Na^+/K^+-ATPase molecules to hydrolyze ATP, thus creating a stimulation of enzyme activity. It increased either by increasing local enzyme concentration on the particle surface, or simply by revealing more active centers. We also propose that the rise in the activity of SPM Na^+/K^+-ATPase may be a consequence of the immobilization of plasma membrane fragments on the surface of the particles. Also, the small surface of the particle in Au NPs might cause the enzyme units to cooperate or perhaps create a local rise in the concentration of substrate and/or cofactors (Mg^{2+}, Na^+ and K^+ ions) necessary for the function of the enzyme, thus facilitating its operation.

5. Conclusion

Spectrophotometric, FTIR and AFM analysis of borate capped Au NPs – SPM assembly indicated that interactions between NPs and proteine molecules occured due to the chemical bonding between NPs surface and positive charged cysteine moiety. The selectivity of the enzyme towards its natural inhibitor, ouabain, was preserved and unchanged. We propose that this rises in enzyme activity (observed through biochemical assays) is a result of chemical bonding and spatial reorganization of SPM on the colloid surface (observed through spectroscopic methods) which induced physical fragmentation and improved distribution of SPM aggregates across the colloid particles (seen on AFM).

Acknowledgements

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