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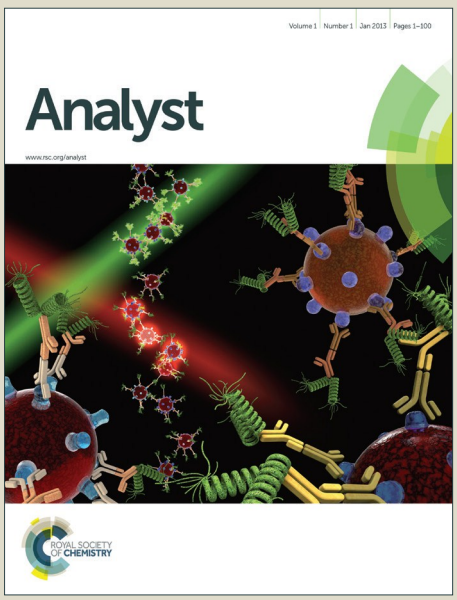

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Picric acid capped silver nanoparticles as a probe for colorimetric sensing of creatinine in human blood and cerebrospinal fluid samples

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Abstract

Creatinine is the most important parameter to be determined in diagnosis of renal, muscular and thyroid function. The most common method for determination of creatinine is Jaffe's reaction in routine practice for blood and urine analysis. But in cases of icteric and haemolyzed blood samples, interference occurs during the estimation of creatinine by other constituents present in blood like bilirubin, creatine, urea, etc., which lead to wrong diagnosis. To overcome such difficulty we have developed a silver nanoparticle (Ag NPs) based sensor for the selective determination of creatinine. Here in this study a new approach has been given to traditional Jaffe's reaction, by coating Ag NPs with picric acid to form an assembly which can selectively detect creatinine. The Ag NPs based sensor proficiently and selectively recognizes creatinine due to ability of picric acid to bind with it and form a complex. The nano assembly and the interactions were followed by transmission electron micrograph (TEM), dynamic light scattering (DLS), UV-Vis, FT-IR and ESI-MS, which demonstrates the binding affinity of creatinine with PA capped Ag NPs. A linear correlation was obtained in the 0.01 μM to 1 μM range with a R^2 value of 0.9998 and lower detection limit of 8.4 nM. The sensor has been successfully applied to different types of blood and CSF samples for determination of creatinine and the results were compared to that of Jaffe's method. With the advantages of high sensitivity, selectivity and low sample volume, this method is potentially suitable for the on-site monitoring of creatinine.

Key words: Picric acid, Jaffe's reaction, Ag NPs, Creatinine, Icteric blood, CSF.

1.0 Introduction

In clinical practice creatinine is one of the most important parameter that is often determined. Concentration of creatinine in blood is an indicator of normal or abnormal renal function, since the concentration of creatinine in blood and urine reflects consistent and closer index of renal glomerular filtration rate [1]. Creatinine is the naturally occurring end product of creatine mechanism and it is less affected by the dietary changes. Thus it has become one of the most important diagnostic indices for renal, muscular and thyroid functions [2, 3]. Also in forensic toxicology for quantitative analysis of therapeutic, illicit drugs and xenobiotic in urine, precise measurement of creatinine is highly important [4].

In adults normal concentration of creatinine in serum and urine is about 5-17 μg/ml, while concentration of creatinine is much lower in children and patients suffering from several diseases [5-7]. But in such cases elevation of concentration of creatinine is very minute which has to be measured very accurately without which false result may lead to wrong diagnosis and cause renal disorder or other complications. In the case of icteric blood, false result of creatinine is found commonly which leads to wrong diagnosis many times. The reason is that, in the course of catabolism of aged RBCs haem is being recycled and produce biliverdin, which is converted in to yellow colored pigment bilirubin. Normally it is excreted in bile and urine [8]. Bilirubin is also an important test to know normal hepatic function. The tests for creatinine and bilirubin are performed simultaneously in renal and hepatic disorder in the same patient for true diagnosis [9]. But in the case of hepatic disorder, level of bilirubin elevate and it gives interference in the absorption of yellow colored picrate used to measure creatinine by the Jaffe's method and thus lead to false result [10]. Due to this, very often critical level of creatinine shows normal concentration in the test performed [11]. Many times prior oxidation of bilirubin is done before test as a sample preparation which is tedious and time consuming. Also due to different reasons like preparative procedure error, mechanical stress, intrinsic RBCs membrane defect, bacterial contamination, temperature, sudden osmotic or pH change, drugs initiate hemolysis and diseases like hemophilia and many others, serum samples turn reddish in color due to presence of free hemoglobin in it. Hemoglobin is present in RBC, but when RBC are being lysed hemoglobin gets separated and appear in serum in its free form [12]. Estimation of creatinine in such haemolyzed serum samples give quasi decreases in result due to alkaline dissociation of free hemoglobin in sub

unit of serum. It also absorbs some part of wavelength and give difference in total absorbance which lead to wrong diagnosis [13].

To overcome this precarious delinquent many scientists have strived to develop highly accurate, easy and quantitative methods to determine creatinine levels in blood and urine, which have a lower detection limit in at least the sub-micro molar range [1]. Among them few of the methods are based on Jaffe's reaction. In 1886 German scientist Max Jaffe developed Jaffe's reaction for detection of creatinine [14-17]. The reaction follows the principle that in alkaline medium picric acid binds with creatinine to form orange complex. This method is cheap and simple. But other bio molecules that are present in blood and urine like bilirubin, creatine, urea, etc. interfere in the reaction and sometimes give imprecise result. Apart from this there are several instrumental techniques like capillary zone electrophoresis [18-20], liquid chromatography- isotope dilution mass spectrometry [21], amperometry [22], high performance liquid chromatography (HPLC) [23-24], electrochemical technique [25], nafion coated copper plating electrode [26] spectrophotometric methods [27], colorimetry [28] and potentiometric sensors [29] and many more which have been employed for detection of creatinine from blood and urine. But almost every technique offers either expensive stationary or needs rigorous pre sample treatment for detection.

Thus in spite of having a large number of methods for the detection of creatinine there is a need of a method which is simple, rapid, cost effective and above all highly accurate which has lower detection limit up to nano molar concentration.

To fulfill such purpose nanotechnology can be used. Nanotechnology has emerged as a newly interdisciplinary and fast growing field. Nanoparticles are having a tremendous growth in the field of medicine and clinical industry due to their chemical and physical properties. Among all silver nanoparticles (Ag NPs) have attracted considerable interest from almost every field due to its unique properties like easy synthesis, high thermal conductivity, high resistance to oxidation, anti-fungal and anti-bacterial activity and so on [30]. Due to its small size and easy to handle property it has rapidly adopted in the medical field. The use of silver nanoparticles in the medical field can be divided in to two main parts i.e., diagnostic and therapeutic. Due to its extra ordinary properties silver nanoparticles can be subjected to detect different bio-molecules present in different body fluids even in nano molar concentration.

Our research group has synthesized and reported a number of nanoparticles based sensors for the detection of different biomolecules [31 – 33]. Inspired by our previous results, we designed a new picric acid (PA) capped Ag NPs for determination of creatinine in different body fluids. The developed sensor provides selective determination of creatinine even in cases like icteric blood, haemolyzed blood and CSF. The orange red colored complex formed due to the interaction of PA capped Ag NPs and creatinine provides ultra-sensitivity and selectivity especially due to the noninterference of bilirubin which often interferes with the estimation of creatinine from blood and urine samples. Thus here in this study a new dynamic approach has been given to silver nanoparticles and an effort has been made to give a novel approach for the accurate detection of creatinine.

2.0 Experimental

2.1 Materials & Method

Silver nitrate ($AgNO_3$, ≥ 99.0%), sodium borohydride ($NaBH_4$, 99.99%), picric acid (2, 4, 6- Trinitrophenol, ≥ 98%), polyvinylpyrrolidone (PVP), creatinine (≥ 98%) and all bio-chemicals were purchased from Sigma Aldrich. Deionized water is used throughout the experiment. All glassware were thoroughly washed by 3:1 HCl: HNO_3 solution and rinsed with deionized water prior to analysis. The absorbance spectra were recorded on Shimadzu UV-1800 UV Visible Spectrophotometer. The FT-IR spectra were recorded on Bruker TENSOR-27 in the range of 4000- 400 cm^{-1} using KBr pellet. ESI Mass spectra were taken on a Shimadzu GCMS-QP 2000A. DLS measurements were performed using Metrohm Nanotrac instrument. TEM micrograms were obtained from Phillips Transmission Electron Microscope 200 KV (TEM).

2.2 Preparation of Nano particles

Silver nanoparticles were synthesized by the reported method [34] by reducing aqueous $AgNO_3$ solution in the presence of $NaBH_4$ and PVP solution. In this typical method, an aqueous solution of $NaBH_4$ (2 mM) was prepared in an Erlenmeyer flask. The solution (15 ml) was stirred in an ice bath for 20 min. Then 2 ml of aqueous solution of $AgNO_3$ (1 mM) was added in to the solution at a constant rate of 1 drop per second. Soon the mixture turned in to light yellow color. In this mixture 1 ml of 1% PVP solution was added. As soon as the solution of PVP was completely added stirring was stopped. Silver nanoparticles (55 μM) were stored in a clean amber colored glass vial until use. Synthesized NPs were stable for 6 months. Ag NPs were characterized by TEM, DLS and UV Visible spectrophotometry.

2.3 Preparation of picric acid capped silver NPs

Freshly prepared recrystallized picric acid (4 ml, 1 mM) is added dropwise in to freshly synthesized Ag NPs (2 ml, 55 μM) and the mixture was stirred for 30 min at room temperature (27º C).

2.4 Preparation of standards and detection of creatinine

The stock solution of 1 M creatinine was prepared by dissolving 1.131 g of creatinine in 10 ml Milli Q water and was diluted as and when required. For detection of creatinine 100 μl of PA capped Ag NPs (18 μM) was mixed with 100 μl of different encoded quantities of standard creatinine (0.01 μM to 1 μM) after adjusting the pH to 7 with phosphate buffer solution and the solution was made up to 2 ml with deionized water. After 4 min incubation at room temperature (27º C) the absorption spectra was recorded at 500 nm against reagent

blank using Shimadzu UV-1800 UV Visible Spectrophotometer. The blank does not absorb at this wavelength. A calibration curve was prepared with the concentration against the absorbance.

2.5 Collection of real samples and analysis

To explore the possibilities of application of the developed sensor to biological samples, number of different real samples of blood were analyzed using the present method. 2 ml of blood samples were collected from healthy individuals, haemolyzed and icteric blood samples and CSF samples were collected from Parikh Pathology laboratory, Ahmedabad with due permissions. Blood samples were collected by percutaneous puncture using a 5 ml sterile syringe attached with 20 gauge needle and blood samples were centrifuged at 2000 rpm prior to analysis and supernatant was used. CSF sample was collected by lumber puncture using a lumber puncture needle of 22 gauge needle attached with 5 ml sterile syringe. All body fluids were stored at 4 º C immediately after collection and analysis was performed within one hour after collection of samples. Blood (100 µl) and CSF samples (100 µl) were directly mixed with PA capped Ag NPs. And absorbance was measured at 500 nm against reagent blank.

3.0 Results and Discussion

Freshly synthesized Ag NPs exhibit yellowish color in aqueous solution due to excitation of surface plasmon resonance in Ag NPs. As the nanoparticles comes in to close proximity with the bio molecule, the interaction takes place due to electrostatic force and hydrogen bonding resulting in the formation of an orange red complex consequently leading to aggregation of the nanoparticles. This color change can be easily distinguished by the naked eyes. Here in this study picric acid capped Ag NPs is used to detect creatinine from the different types of body fluids. Picric acid capped Ag NPs displays a high stability at room temperature (27 ºC) without any aggregation. For the characterization as well as to ascertain the interaction between creatinine and PA capped Ag NPs UV Visible spectrometry, FT-IR, ESI Mass, DLS and TEM analysis were carried out. The details are given below.

3.1 UV visible spectrometry

Most of the applications of Ag NPs as sensor are based on detecting the shift in surface plasmon peak. This shift may be due to the aggregation of Ag NPs induced by the analyte. Here we investigated spectrophotometrically the absorption spectra of the Ag NPs solution, which shows an absorption maximum at 410 nm. After several months of storage, the Ag NPs surface plasmon band wavelength remained the same which indicates that NPs are stable. The solution of PA capped Ag NPs without creatinine is of fluorescent yellow color due to the presence of picric acid in the system and it displayed an intense surface plasmon band at about 395 nm, while in presence of 1 μM creatinine the wavelength of maximum absorption (λmax) of Ag NPs shifted from 395 nm to 500 nm and the color of Ag NPs changed from fluorescent yellow to dark orange which indicate the state of interaction with the biomolecule (Fig. 1 (A), (B) and (C)). The blank nanoparticles did not show any absorption at 500 nm.

The specific change in spectral profile was noticed by gradual addition of different concentrations of creatinine from 0.01 μM to 1 μM with PA capped Ag NPs (Fig. 2). Also it was found that even lower concentration of creatinine gives a color change which can be easily distinguish by naked eyes. However color intensity decreases after 4 days due to sedimentation.

3.2 Linearity and detection limit

Figure 2 & 3 shows linearity curve of picric acid capped Ag NPs with different concentration of creatinine. The linearity curve shows that the present developed method is linear from 0.01 μM to 1 μM, with R^2 value of 0.9998. The regression equation is y =

1.0068x + 0.0036 where, y is the absorbance and x is the concentration of creatinine. The lower detection limit (LOD) is 8.4 nM according to 3σ IUPAC criteria.

3.3 The effect of the reaction time

The absorption of PA capped Ag NPs remained unchanged after 4 min which indicates that the reaction is completed in 4 min and hence, all absorption measurements were made 4 min after addition of creatinine (Fig. 4).

3.4 Selectivity study

The selectivity of the assay was carried out with other analytes which are normally present in human blood and with some metal ions which may interfere in the reation. The analytes alanine, arginine, aspartic acid, cysteine, 3-(3,4-Dihydroxyphenyl)-DL-alanine, glutamic acid, glycine, histidine, hydroxyproline, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, creatine, urea, ascorbic acid, citric acid, glucose, bilirubin, fructose, lactose and glutathione at a concentration of 10 μM were used for the detection of 10 μM creatinine in the presence of PA capped Ag NPs, while for metal ions different cations and anions like Fe^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Li^{+}, Na^{+}, Br^{-}, SO^{4-} were used at a concentration of 10 μM. The results are shown in Figure 5, 6, 7 and 8. It clearly indicates that creatinine showed a higher absorption which confirmed the selectivity of the proposed assay for creatinine.

3.5 Effect of pH

We examined the stability of PA capped Ag NPs creatinine complex at different range of pH conditions. From that it is concluded that PA capped Ag NPs creatinine complex is stable in the range of 5 to 7, for weeks to months and no sign of further aggregation were observed.

3.6 Dynamic light scattering measurements

For the evaluation of aggregation of PA capped Ag NPs with creatinine, we employed DLS analysis of the resultant product. It is illustrated that before capping of picric acid, the Ag NPs had an average hydrodynamic diameter of ~20 nm, which maintain their size and stability. After capping with picric acid the average diameter becomes ~34 nm which is close to the diameter of Ag NPs. In contrast, upon addition of creatinine the average diameter increased to ~1100 nm (Fig. 10).

3.7 TEM analysis:

TEM measurements were studied to evaluate the aggregation of PA capped Ag NPs – creatinine complex further. The TEM micrographs were taken for typical samples of Ag NPs,

PA capped Ag NPs and PA capped Ag NPs with creatinine. Ag NPs shows a mean diameter of ~ 15 nm (Fig. 11 (A)), while PA capped Ag NPs shows a mean diameter of ~ 30 nm (Fig. 11 (B)). On addition of 1 μM creatinine into PA capped Ag NPs, the TEM measurements indicates aggregation due to the PA capped Ag NPs – creatinine complex formation (Fig. 11 (C)).

3.8 FT-IR Spectra

The FT-IR spectra of picric acid, PA capped Ag NPs and PA capped Ag NPs-creatinine complex is shown in Figure 12. All the FT-IR spectra are taken in the region of 4000 to 400 cm^{-1}. The most notable modes from picric acid are the Ar-O-H stretching sharp band at 3107 cm^{-1}, two Ar-NO_2 stretching bands at 1561 and 1369 cm^{-1} and N-O symmetric stretching band at 1280 cm^{-1} (Fig. 12 (A)). The spectra of Ag NPs bound to picric acid show broad –O-H peak at 3188 cm^{-1} and -NO_2 peak shifted to 1300 cm^{-1}, which suggest that the picric acid coordinate with the silver atoms on the surface of Ag NPs through –OH and NO_2 groups. Fig. 12 (C) shows a new peak of –C=O stretching at 1755 cm^{-1} due to five membered ring, while broad –N-H/ -O-H stretching band at 3200 cm^{-1} is due to H – bonding between creatinine and nanoparticles. The Ar-C-N stretching at 1332 cm^{-1} and N-H bending of 2^{o}-amine at 935 cm^{-1} were also observed. These observations confirm the formation of picric acid capped Ag NPs-creatinine complex via electrostatic interaction and intermolecular hydrogen bonding.

3.9 ESI MASS Spectra

The interaction between PA capped Ag NPs and creatinine was further studied by ESI-MS spectroscopy. Fig. 13 Shows the ESI-MS spectra of the pure picric acid (Fig. 13 A), PA capped Ag NPs (Fig. 13 B) and PA capped Ag NPs with creatinine (Fig. 13 C) in aqueous solution. The spectra of pure picric acid showed peak at m/z =230.5, while the Ag NPs capped with picric acid showed peak at m/z =336.2, (Fig. 13 (A) & (B)) that indicates formation of PA capped Ag NPs in aqueous media. Fig. 13 (C) shows the ESI MS spectra of the PA capped Ag NPs with creatinine in aqueous solution gave the molecular ion peak at m/z = 448.9 (PA + Ag NPs + Cr) and m/z = 450.1 ($[PA + Ag NPs + Cr]^{+}$) which manifestly indicates that the formation of picric acid-Ag NPs-creatinine 1:1 complex through electrostatic interaction and hydrogen bonding.

3.10 Binding mechanism of PA capped Ag NPs with creatinine

The synthesized Ag NPs are stable for 6 months. Ag NPs are of pale yellow in color, when picric acid is coated on Ag NPs a color change was observed from pale yellow to fluorescent yellow. Picric acid is attached with Ag NPS through -OH and -NO_2 groups that

stabilizes the PA capped Ag NPs in a dispersed state. On addition of creatinine due to the tautomeric form of creatinine [35], electrostatic interaction and hydrogen bonding occur between $–NO_2$, -NH, -N groups of picric acid and creatinine and results in a color change from fluorescent yellow to dark orange (Fig. 14). The addition of creatinine aggregates the PA capped Ag NPs and this aggregation results in a color change. A bathochromic shift was observed in the absorbance spectra, with the wavelength of maximum absorption shifting from 395 nm to 500 nm.

3.11 Detection of creatinine in different types of human serum and CSF

To validate the reliability of the present method, we analyzed creatinine in three different types of human serum i.e., icteric blood, haemolyzed blood and normal blood and CSF by the standard addition method (Table 1). The recovery is in the range of 96.7 to 99.9 showing reliability and accuracy of the developed method. A comparison was also made between the results obtained by the present method and that of dry chemistry method [36]. The results of that are presented in Table 2. Also comparison of present method with other reported methods for creatinine estimation were also made and shown in Table 3. Among all methods present method is most selective and accurate and provide reliable result even in low levels and disease conditions.

4.0 Conclusion

Determination of creatinine concentration is very important task in clinical practice. Accurate measurements are very important as false results may lead to wrong diagnosis and can result in complications. To overcome such conditions we have developed a simple, easy and rapid colorimetric probe for the detection of creatinine. The PA capped Ag NPs allowed a rapid detection of creatinine and this probe is highly selective and sensitive. A linear correlation was obtained in the 0.01 μM to 1 μM range with a R^2 value of 0.9998 and lower detection limit of 8.4 nM. The present method is easy, simple, rapid and accurate. It also gives true measurements in cases like icteric and haemolysed blood and other body fluids which make the method more appropriate for routine analysis. The method is based on colorimetric detection which makes it applicable to clinical as well as toxicological analysis. The present method is simple, inexpensive and highly selective than the other published methods.

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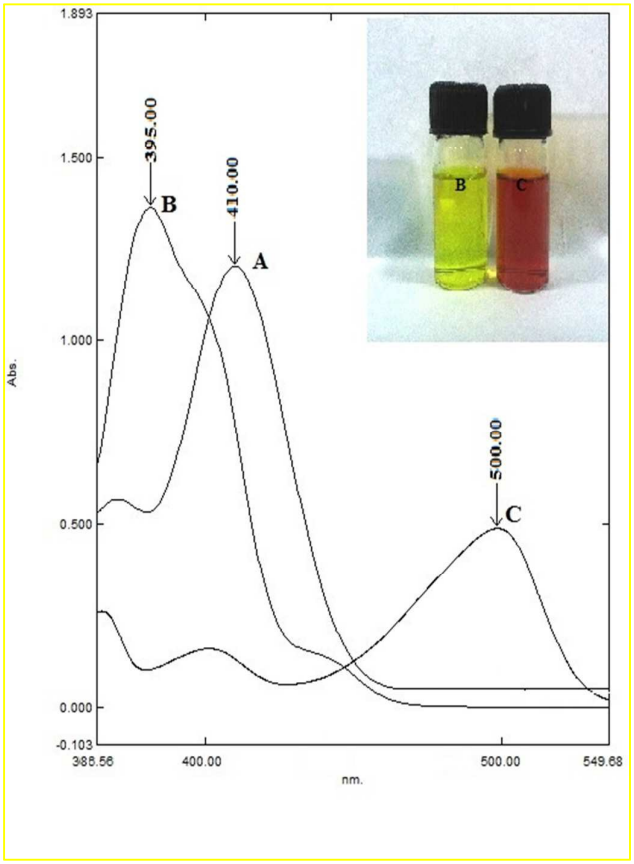


Figure 1 (A) Absorbance spectra of Ag NPs, (B) Absorbance spectra of PA capped Ag NPs, (C) PA capped Ag NPs + creatinine.

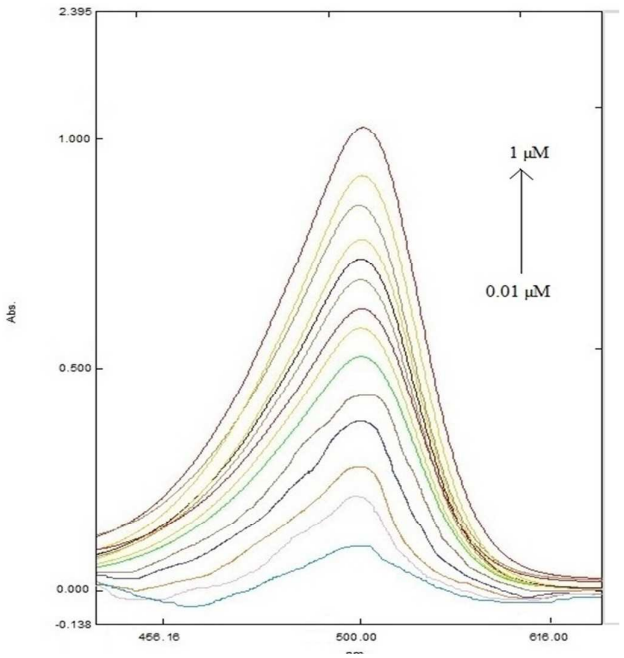


Figure 2 Absorbance spectra of PA capped Ag NPs in the presence of different concentration of creatinine (0.01 µM to 1 µM).

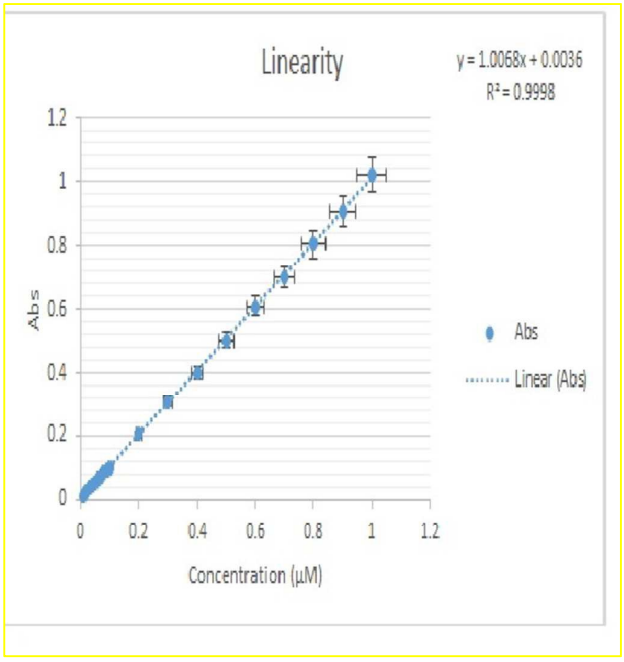


Figure 3 linearity curve of creatinine at pH 7, reaction time 4 min, λmax = 500 nm

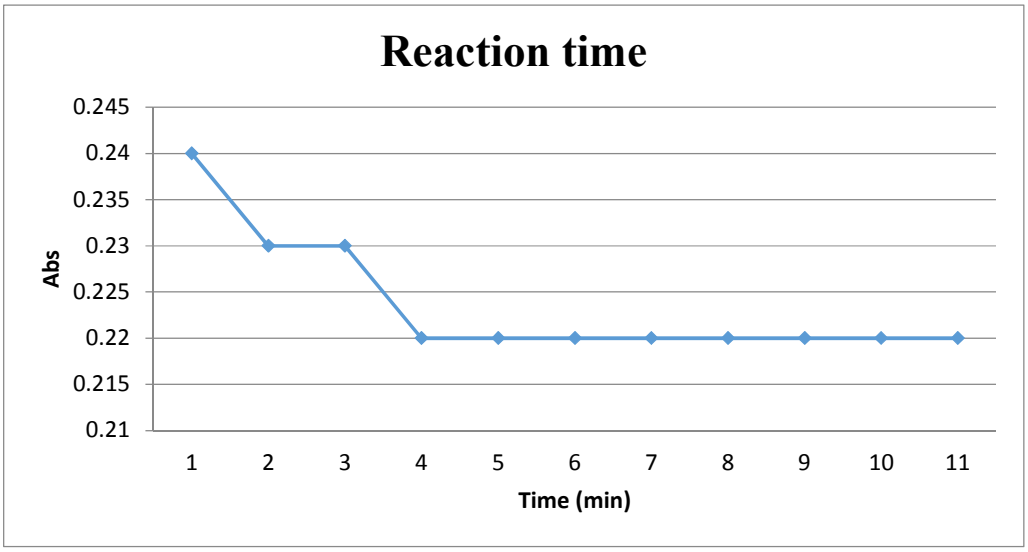


Figure 4 Optimization of Reaction time

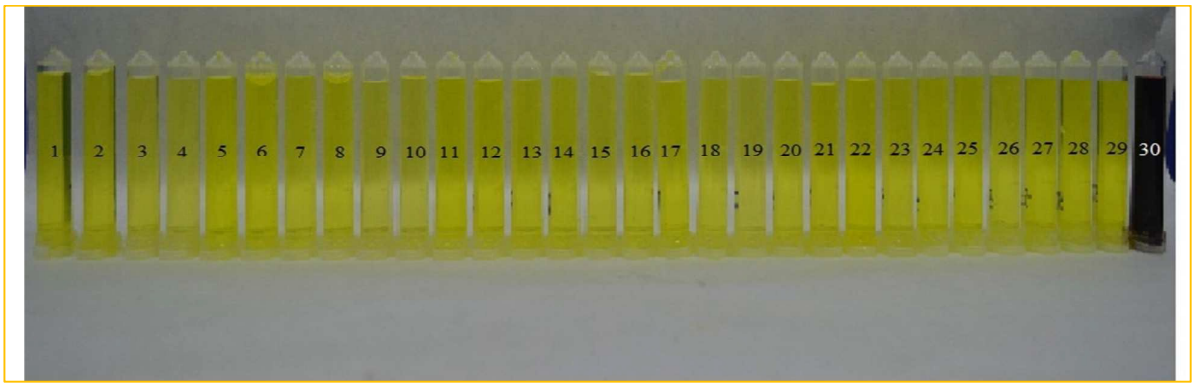


Figure 5 Selectivity of PA capped Ag NPs with biomolecules at a concentration of 10 μM

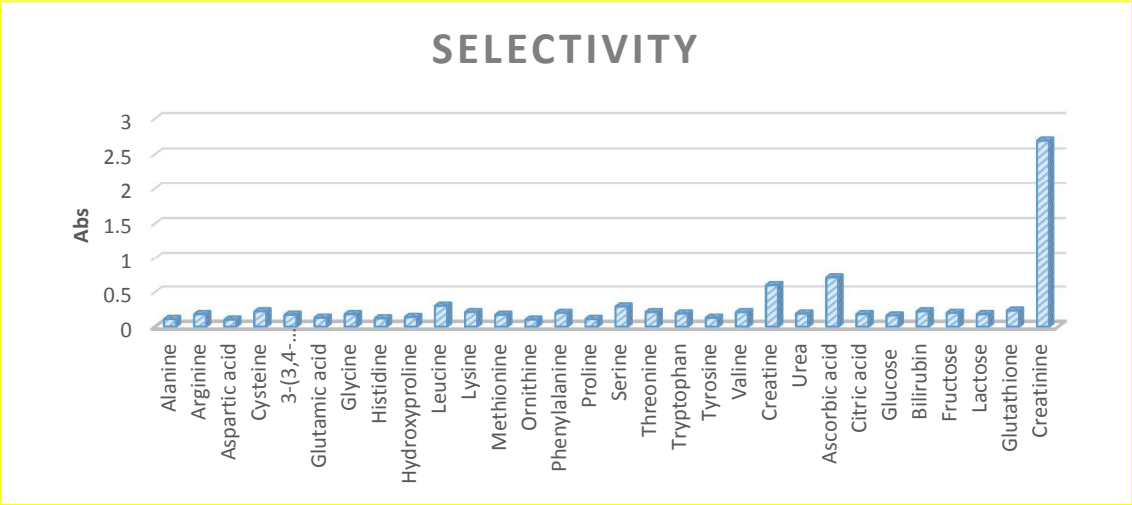


Figure 6 Selectivity of PA capped Ag NPs with biomolecules at a concentration of 10 μM

Figure 7 Selectivity of PA capped Ag NPs with metal ions at a concentration of 10 µM

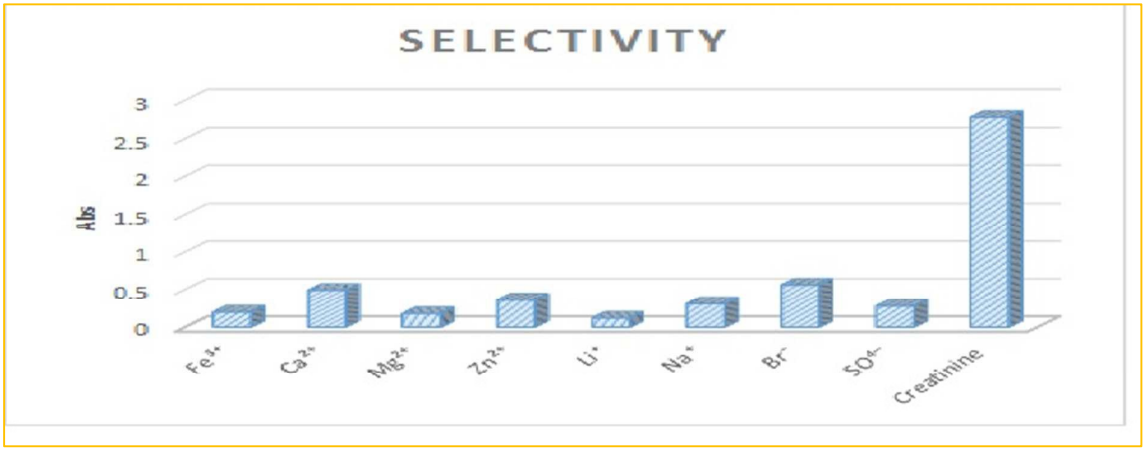


Figure 8 Selectivity of PA capped Ag NPs with metal ions at a concentration of 10 µM

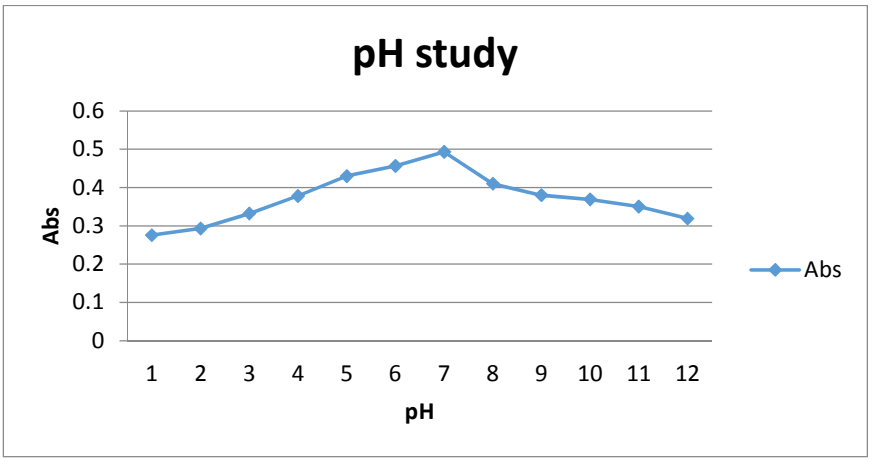


Figure 9 Effect of pH on PA capped Ag NPs creatinine complex

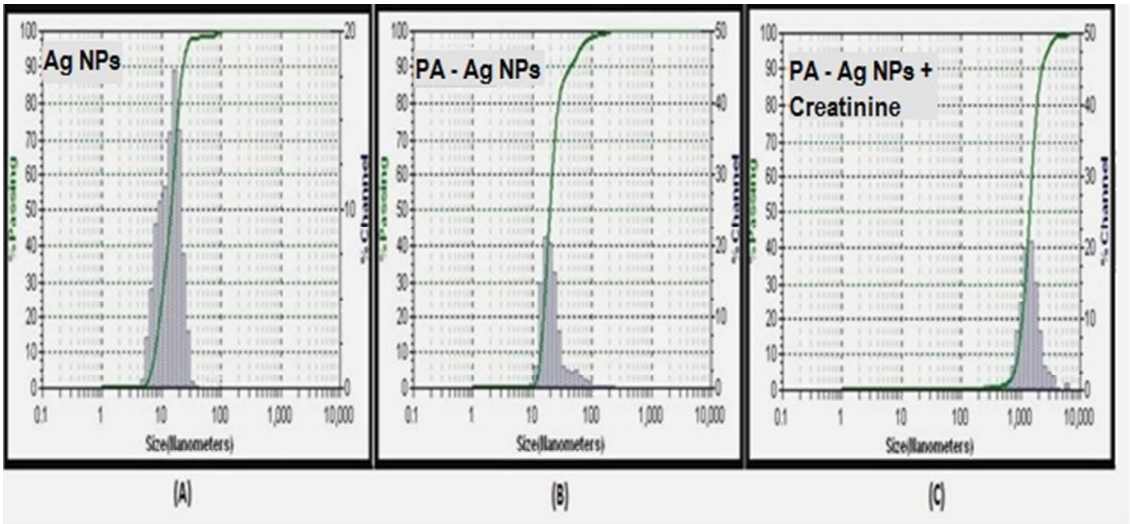


Figure 10 (A) Size distribution of Ag NPs measured by using DLS, (B) Size distribution of PA capped Ag NPs, (C) Size distribution of PA capped Ag NPs in the presence of creatinine

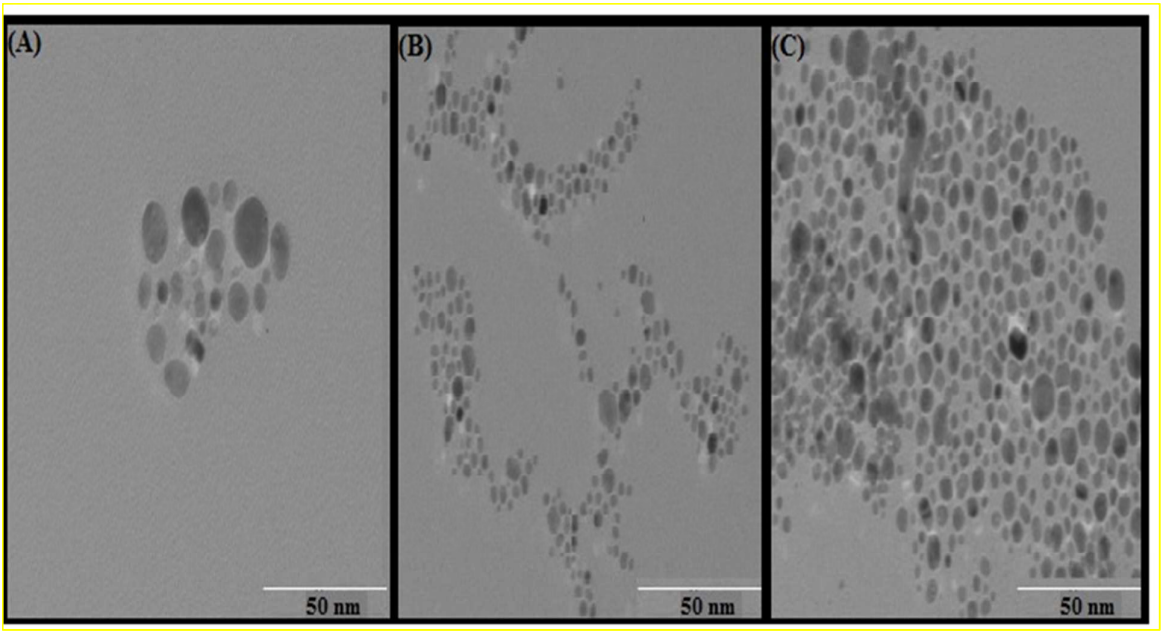


Figure 11 (A) TEM micrograph of Ag NPs, (B) TEM micrographs of PA capped Ag NPs and (C) TEM micrographs of PA capped Ag NPs with creatinine

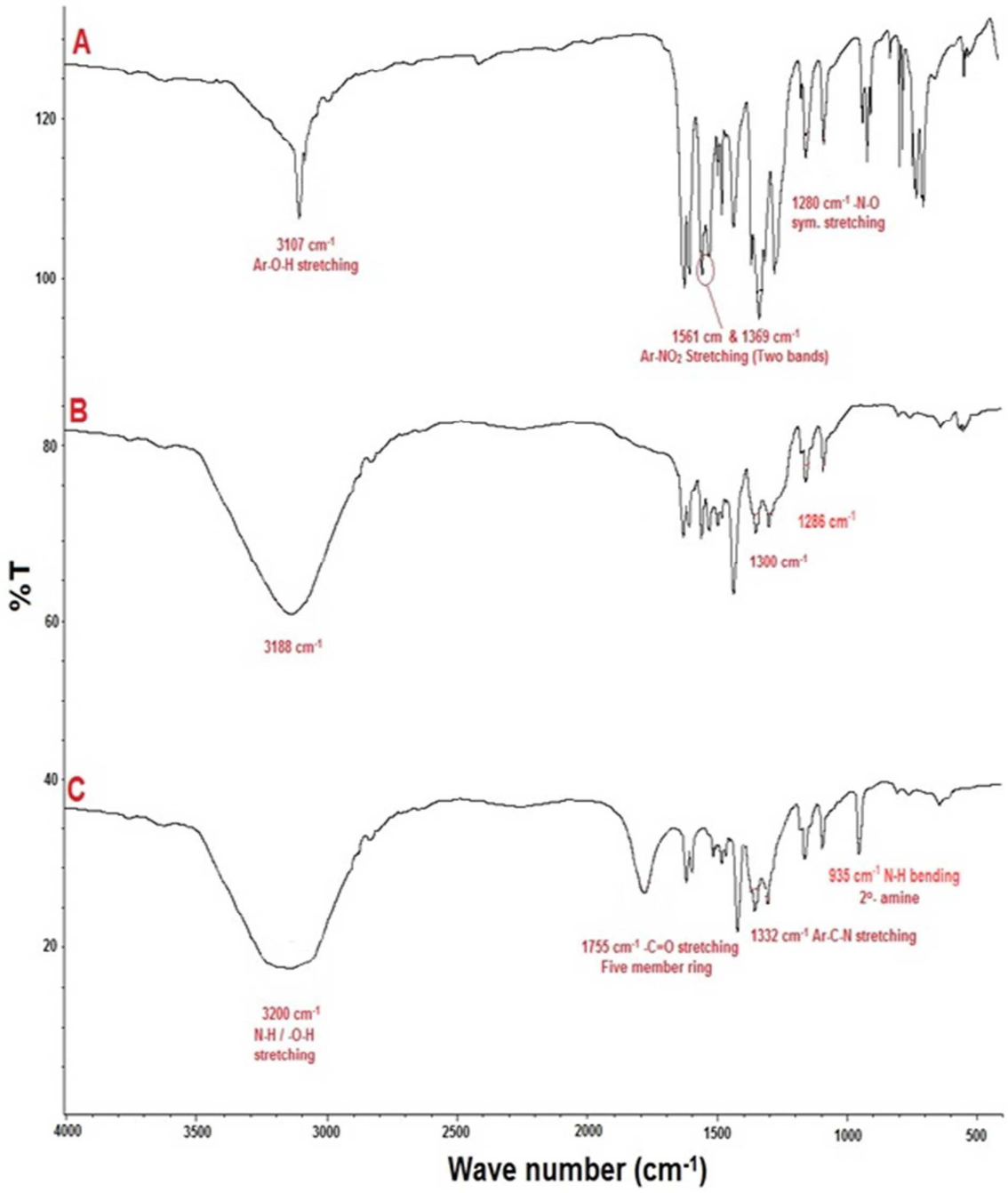
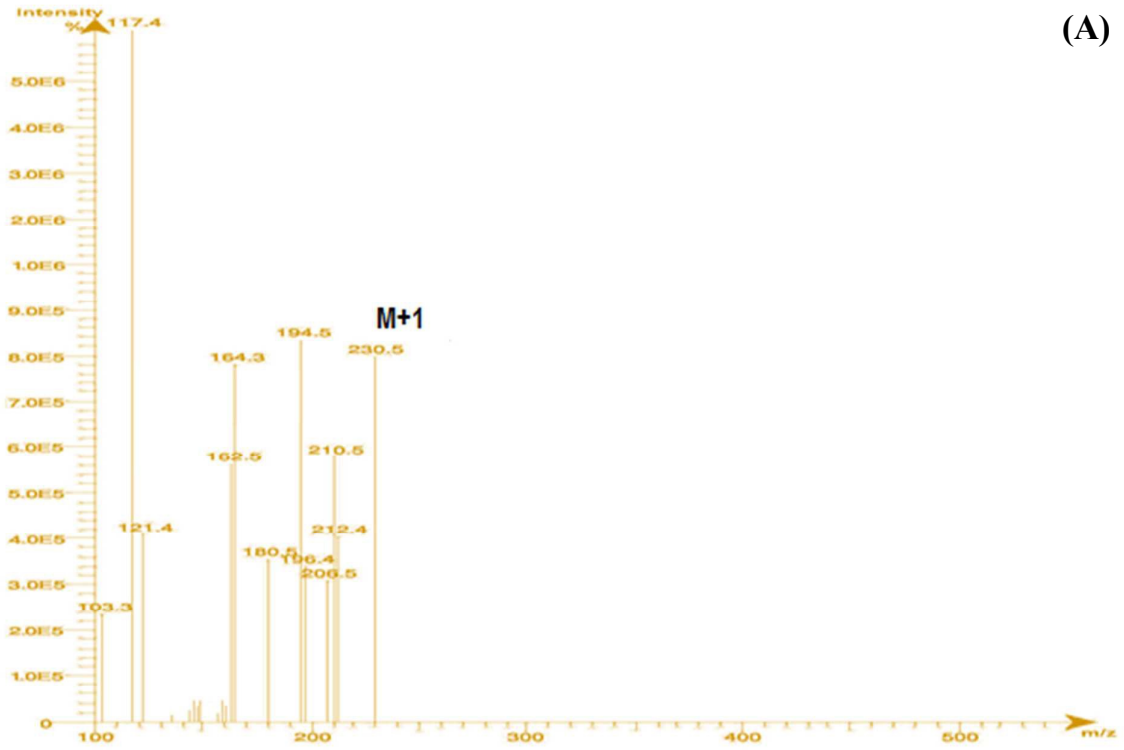
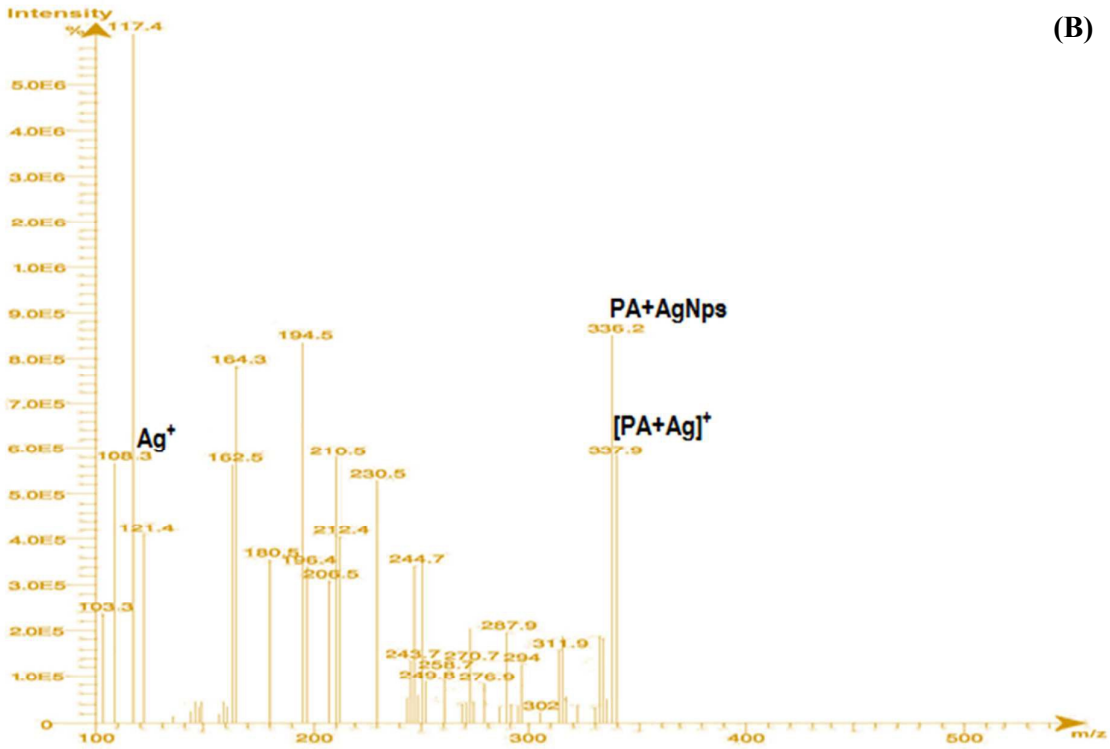


Figure 12 FT-IR spectra of (**A**) picric acid, (B) PA capped Ag NPs and (**C**) PA capped Ag NPs-creatinine complex.





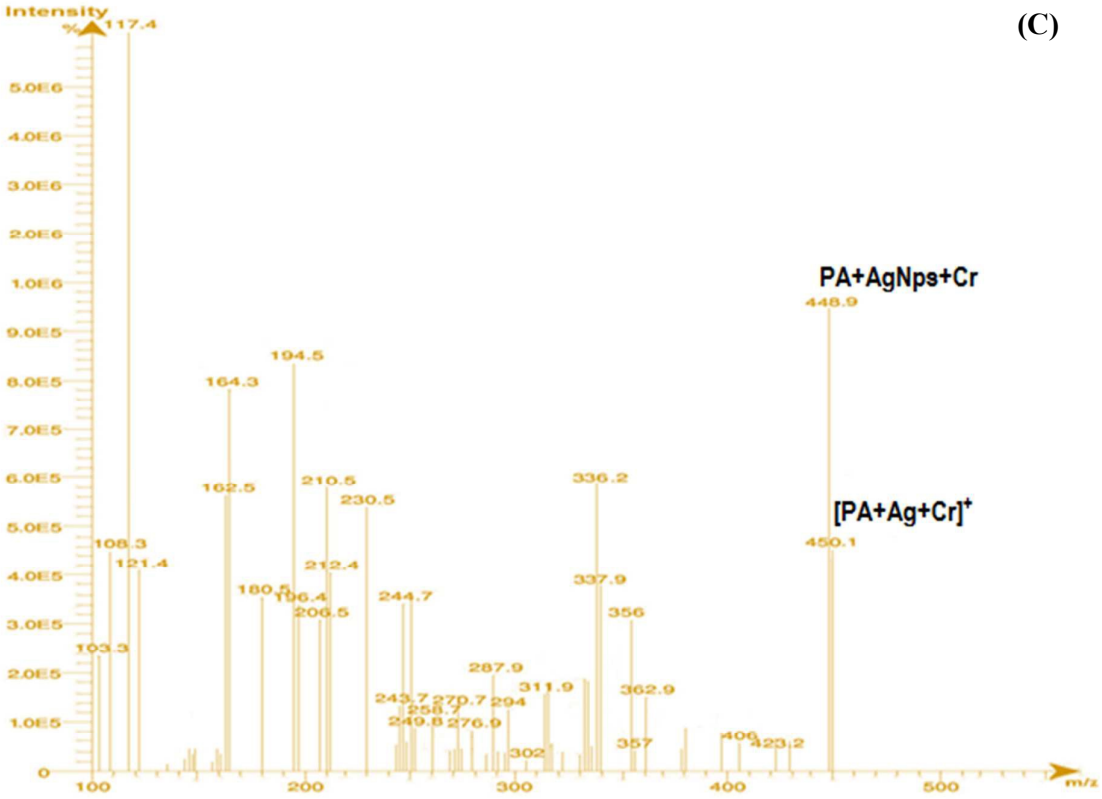


Figure 13 ESI-MS spectra of the picric acid (A), PA capped Ag NPs (B) and PA capped Ag NPs - creatinine (C).

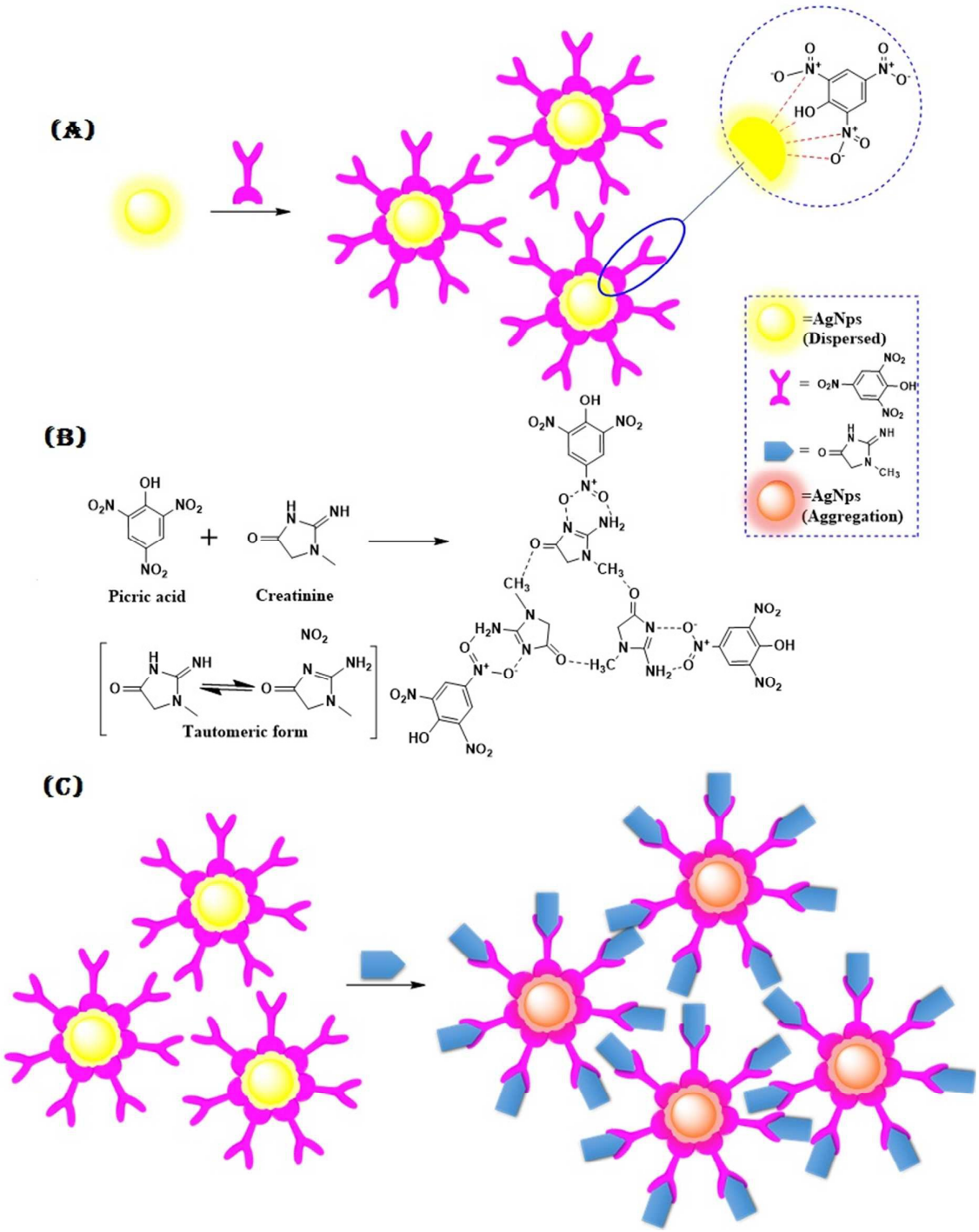


Figure 14 Schematic illustration of proposed mechanism for the colorimetric detection of creatinine.

Table 1 Validation of the developed method for determination of creatinine in different types of blood and CSF samples

Nature of sample	Creatinine estimated by this method (nM)	Addition of creatinine (nM)	Total Creatinine after addition. (nM)	Recovery (%)	Standard deviation of total creatinine after addition
Normal Human Blood Serum	82	100	176	96.70	5.51
Icteric Human Blood Serum	52	100	148	97.36	6.65
Haemolyzed Human Blood Serum	38	100	135	97.20	4.04
Human Cerebrospinal Fluid (CSF)	21	100	120	99.90	3.78

Table 2 Comparison of results obtained from present method and dry chemistry method

Nature of sample	Dry chemistry method (nM)	Present method (nM)
Normal Human Blood Serum	90	82
Icteric Human Blood Serum	56	52
Haemolyzed Human Blood Serum	41	38
Human Cerebrospinal Fluid (CSF)	20	21

Table 3 Comparison of present method with some of the previously published methods

Sr No.	Method	Detection limit	Drawbacks	Ref. No.
1	An improved electrochemical creatinine detection method via a Jaffe-based procedure	0.27 mM	The detection limit is higher	37
2	Rapid and direct determination of creatinine in urine using capillary zone electrophoresis	0.05 mM	The procedure is tedious and time consuming. Also method is not applicable to all body fluids like CSF and Vitreous humor as such fluid contains very small amount of creatinine, while this method have limit of detection quite high.	4
3	A novel method for rapid determination of creatinine in urine by microchip electrophoresis with light emitting diode induced fluorescence detection.	2.87 μmol L^{-1}	Procedure requires pre sample treatment which is time consuming.	38
4	Detection of urinary creatinine using gold nanoparticles after solid phase extraction	13.7 mg L^{-1}	The method and sample preparation procedure is tedious and time consuming	39
5	Highly selective and sensitive photometric creatinine assay using silver nanoparticles	0.003 μmol L^{-1}	Preparation of Ag NPs is time consuming and Ag NPs are having short term stability. The interference of bilirubin not studied.	35
6	Present method	0.0084 μM	The procedure is easy, simple and rapid. The method is highly selective and no interference of bilirubin was observed even in case of icteric blood sample. Method can be applied to any body fluids	-

			for quantitative analysis of creatinine as the detection limit is very less. Method can be used for on the spot detection of creatinine due to the color change property.	