ORIGINAL ARTICLE

An optimal method for measuring biomarkers: colorimetric optical image processing for determination of creatinine concentration using silver nanoparticles

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Abstract

Creatinine concentration is one of the important elements in the body for diagnosing kidney failure, muscular dystrophy, glomerular filtration rate, and diabetic nephropathy. The disadvantages of recently introduced analytical techniques, such as Jaffe's, spectroscopic, colorimetric, and chromatographic methods, for quantifying creatinine in urine involve toxicity, the high cost, interference, and the complexity of the design. In this paper, we designed and fabricated a new colorimetric assay for the measurement of creatinine concentration based on color differentiation generated by mixing different concentrations of creatinine with synthesized silver nanoparticles (AgNPs) coated with polyvinylpyrrolidone (PVP) and polyvinyl alcohol (PVA). An isolated box is designed for the uniform optical imaging of solutions, the captured images are processed in real time, and the quantitative and qualitative results are displayed. For colorimetric processing, a variety of color systems, such as RGB (red, green, blue), CMYK (cyan, magenta, yellow, black), and grayscale (Gr), have been evaluated, indicating that the combination of green (G) and grayscale (Gr) provides the best results for this experiment. TEM analysis and spectroscopy were used to confirm the results of the experiment. Linear range and limit of detection (LOD) were obtained for AgNPs/PVP 0.03–1 mg/dl and 0.024 mg/dl and for AgNPs/PVA 0.01–1 mg/dl and 0.014 mg/dl, respectively, indicating the superiority of our proposed method over recently introduced methods. In this experiment, the detectable resolution with AgNPs/PVP is 40, while it is 71 with AgNPs/PVA. The designed system is simple to use, small in size, and cost-effective for measuring creatinine concentration, while it can be used as a portable system.

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Abbreviations

AgNPs Silver nanoparticles
LOD Limit of detection
TEM Transmission electron microscope
PVP Polyvinyl pyrrolidone
PVA Polyvinyl alcohol
MIP Molecular imprinted polymer
SD Standard deviation
RSD Relative standard deviation

Introduction

Creatinine ($C_4H_7N_3O$) is a waste and non-toxic molecule in the body produced by muscle metabolism in the body. Creatinine concentration is one of the important elements in the body for diagnosing kidney failure, muscular dystrophy, glomerular filtration rate, and diabetic nephropathy (Zinchenko et al. 2012; Gao, Li, and Zhang 2010; Zamora et al. 2007; Kasap et al. 2017). After production, creatinine is transferred to the kidneys and is excreted through the urine without reabsorption in the tubular structures (Jalili and Khataee 2019). Each day, 2% of creatine is converted to creatinine with a constant rate, which is an irreversible and spontaneous conversion. Therefore, the level of creatinine concentration in the body can be measured in blood and urine samples (Iseki et al. 1997; Barr et al. 2004). Under normal conditions, the level of creatinine in the blood serum is between 35 and 140 mMl (Mohammadi and Khayatian 2015). Nowadays, Jaffe's method is routinely used in clinics to determine the level of creatinine. In this method, creatinine reacts in an alkaline medium with picric acid, and the change in the color of the resulting solution (yellow to orange) can be measured by a spectrophotometer (Delanghe and Speeckaert 2011). Interference of picric acid with other biomolecules, such as glucose, urea, uric acid, and ascorbic acid, decreases the sensitivity and selectivity of Jaffe's method (Du et al. 2016). Moreover, the toxicity of picric acid limits the application of this method in clinical settings. Furthermore, a number of different methods, such as spectroscopic (Krishnegowda et al. 2017), electrochemical (Diouf et al. 2017), colorimetric (Sununta et al. 2018; Du et al. 2015), and chromatography (Zhao 2015), have been developed for determining the concentration of creatinine.

While enzyme-based methods are highly selective and have a good sensitivity, and chromatographic methods provide good accuracy and have less interference (Hewavitharana and Bruce 2003; Mohabbati-Kalejahi et al. 2012; Zakalskiy et al. 2019), these methods often require massive and expensive equipment, various substances to be prepared, skilled personnel, and expensive materials (Mohammadi and

Khayatian 2015). Moreover, colorimetric-based methods have a cost advantage and are easy to use; however, these methods face difficulties in terms of quantitative concentration measurements. To overcome this big challenge, plasmatic nanoparticles (gold and silver) have a good potential due to a number of properties, such as cost-effectiveness, high ability for surface reaction, high absorption ability, and large S–V (surface-to-volume) ratio, leading to improved catalytic performance and optical properties (Du et al. 2013; Yadav et al. 2012; Kasap et al. 2017). Furthermore, NPs are also used to enhance the response of enzyme-based and molecular imprinted polymer (MIP)-based biosensors (Nouira et al. 2012; Yadav et al. 2011,2012), as well as to directly determine the creatinine concentration with spectrophotometric and colorimetric methods (He et al. 2015; Mohammadi and Khayatian 2015; Huang et al. 2015). Therefore, it is essential to develop a cheaper, easier, more effective, and more accessible method to determine the concentration of creatinine.

In this study, we introduce a simple, cheaper, portable, and more efficient method for determining creatinine levels based on synthesized nanoparticles and colorimetric image-processing techniques. For this purpose, PVP-coated silver nanoparticles (AgNPs@PVP) and PVA-coated silver nanoparticles (AgNPs@PVA) were synthesized. Different concentrations of creatinine will create color differentiation when mixed with the synthesized solution. The color changes, as an indicator for creatinine levels, were measured from images captured in a designed isolated box with a uniformly illuminated imaging environment. The captured images were processed in real time by MATLAB software application, and the results compared with those of the spectroscopic-based method.

Material and methods

Materials

All chemicals and solvents used in this experiment were of an analytical grade and of highest purity, and they were used as received without additional purification. Moreover, double-distilled and deionized water was used to prepare the solutions in the experiments to prevent possible interference. Creatinine ($\geq 99\%$), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA) 72,000, silver nitrate (AgNO3, $\geq 99.0\%$), and tri-sodium citrate dehydrate ($\geq 99.0\%$) were purchased from MERCK Company (https://www.merck.com). The glassware used was thoroughly washed with aqua regia, then rinsed thoroughly with deionized water, and finally air-dried prior to use.

Instrumentation

Ultraviolet visible spectrometer was used to measure and record the absorption spectra in the samples. To obtain the size and aggregation of the nanoparticles, we used a transmission electron microscope (TEM ZEISS EM900) at the Molecular Medicine Research Center (Tabriz University of Medical Sciences, Tabriz, Iran). We also used an imaging isolation box developed by the authors to measure the color changes in the samples.

Synthesis of silver nanoparticles with PVP

To synthesize silver nanoparticles with PVP, we used sodium borohydride, NaBH4, as the reducing agent and PVP as the stabilizing agent (Van Dong et al. 2012; Parmar et al. 2016). For this purpose, 3 mg of NaBH4 powder was dissolved in 45 ml of deionized water and then poured into a glass conical flask. Afterward, it was put in an ice bath and stirred for 25 min. Thereafter, 6 ml of 3 mM AgNO3 aqueous solution was added slowly to the solution with gentle mixing. Immediately after, 3 ml of 1%w/v PVP solution was added to the solution and stirring was stopped. The color of the obtained solution is yellow. This solution is kept in an amber glass bottle.

Because the concentration of nanoparticles affects the colorimetric results, the values obtained from the experiments should not be increased or decreased. To ensure a complete reaction and the removal of excess particles, we use more reducing agent than silver nitrate so that silver nitrate is completely converted into silver nanoparticles, and this is in fact our limiting reactor. Also, to remove excess nanoparticles, after synthesis, the solution is centrifuged and the excess amount separated from the main solution.

Synthesis of silver nanoparticles with PVA

This synthesis is similar to the previous one, except that PVA is used here instead of PVP. For this purpose, 45 ml of 1.8 mM aqueous solution of NaBH4 was poured into a glass conical flask and then placed in an ice bath on stirrer for 25 min. Then, 6 ml of 2 mM AgNO3 solution was slowly added. Afterward, 3 ml of 2% PVA solution was added. The obtained light yellow solution was kept in an amber glass bottle until use.

Sample preparation and general procedures

In Blackburn et al. (Alula et al. 2018) and Blackburn et al. (Parmar et al. 2016), it was found that adding creatinine to silver nanoparticles caused aggregation and discoloration in the solution. We used these findings to measure the creatinine concentration by image processing in real time.

Polyvinyl pyrrolidone (PVP) is also used as a reducing, stabilizing, dispersing, and shape controlling agent in the synthesis of silver nanoparticles (Wang et al. 2005; Koczkur et al. 2015). The mechanisms of polyvinyl pyrrolidone in the preparation process were discussed through the optical characteristics of the reaction system (Wang et al. 2005). PVP attached to the silver and protected the Ag particles from agglomerating and growing. Moreover, polyvinyl alcohol (PVA) with the chemical formula of $(C_2H_4O)_x$, a water-soluble synthetic polymer, is a reducing agent for silver nanoparticles. The solution synthesized in reaction with creatinine causes discoloration in the sample (Fig. 1) (Lad 2012).

To perform the experiment, we created different creatinine concentrations using creatinine powder (0–100 mg/dl). When testing silver nanoparticles coated with PVP, the solution pH was adjusted to 6.5 (using NaOH). Then, 200 µl of different concentrations of creatinine was added to 1 ml of the solution (at 27 °c). After 30 min, the color of the solution stabilized, and image processing and spectrophotometric tests were performed on the samples. Moreover, for testing silver nanoparticles coated with PVA, the pH of the solution was first adjusted to 7.5. Afterward, we mixed 400 µl of different concentrations of creatinine with 1 ml of the solution at 35° C. After 20 min, the color of the solution stabilized, and image processing and spectrophotometric tests were performed on the samples.

Results

The imaging box was designed to isolate the ambient light, fix the light and temperature inside the box, and to generally obtain an accurate measurement of the creatinine concentration. After preparation, imaging of the samples was performed using a cuvette placed in the box, and then the images were sent to the system in real time for processing. In this method, the processing is based on the color changes in the samples caused by different concentrations of creatinine. Figure 2 depicts the schematic diagram of the proposed imaging system. For comparison, spectroscopy of the samples was simultaneously performed using a spectroscope.

Moreover, TEM analysis is also used to confirm the results of the experiment. TEM imaging was used to capture fine details—even as small as a single column of atoms, which is thousands of times smaller than a resolvable object seen in a light microscope (Fig. 3). The TEM imaging results indicated that adding creatinine to both samples containing silver nanoparticles with PVP and PVA resulted in the aggregation of the particles and discoloration in the solution.

To measure creatinine concentration, solutions with different concentrations of creatinine were prepared and
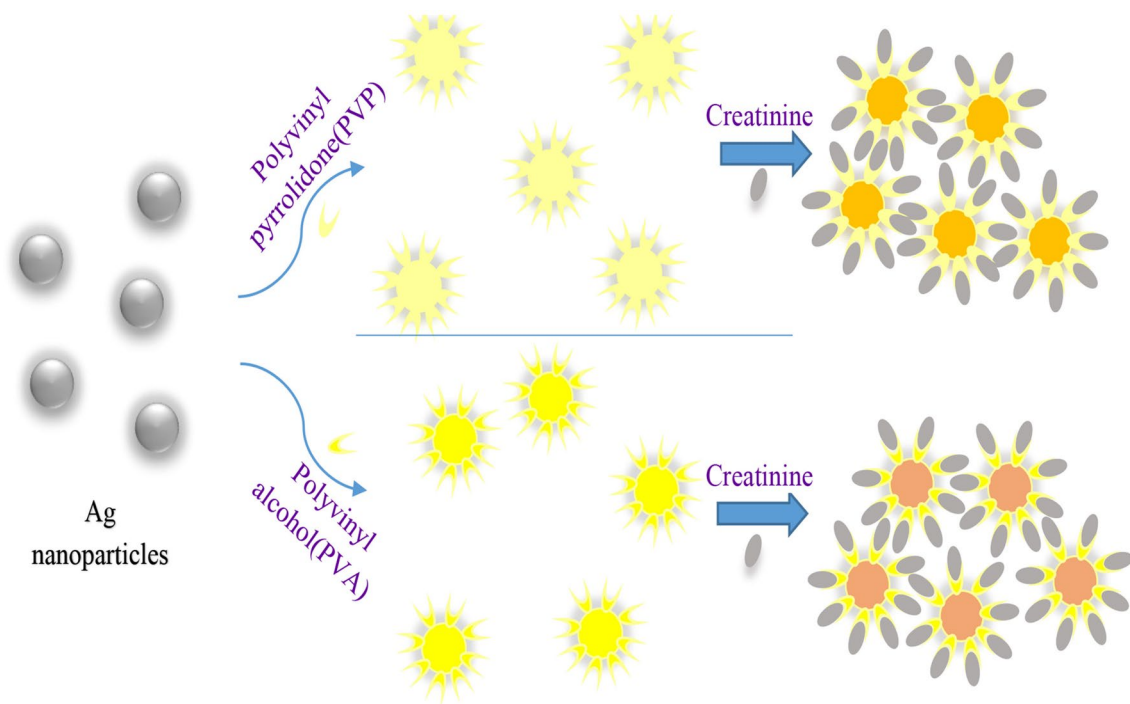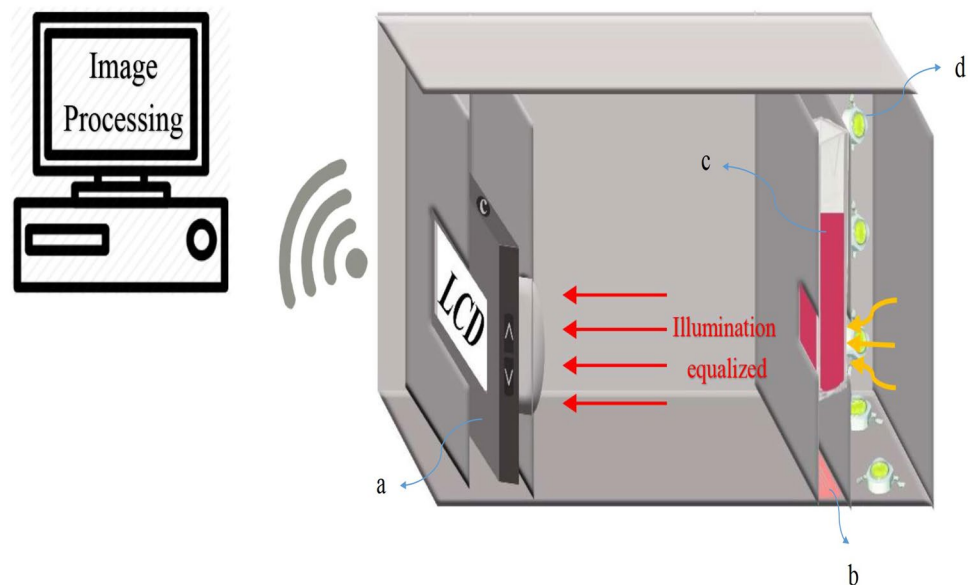
Fig. 1 Schematics of process of AgNPs/PVP and AgNPs/PVa with the creatinine and the color change obtained in the interaction with AgNPs

Fig. 2 Colorimetric optical image-processing box components: a: Camera, b: thermal element and temperature control module. c: Sample cuvette. d: Power LED. The illumination equalized in the box reaches the imaging device after passing through the sample. The captured image is transferred to the real-time processing system and the results are displayed
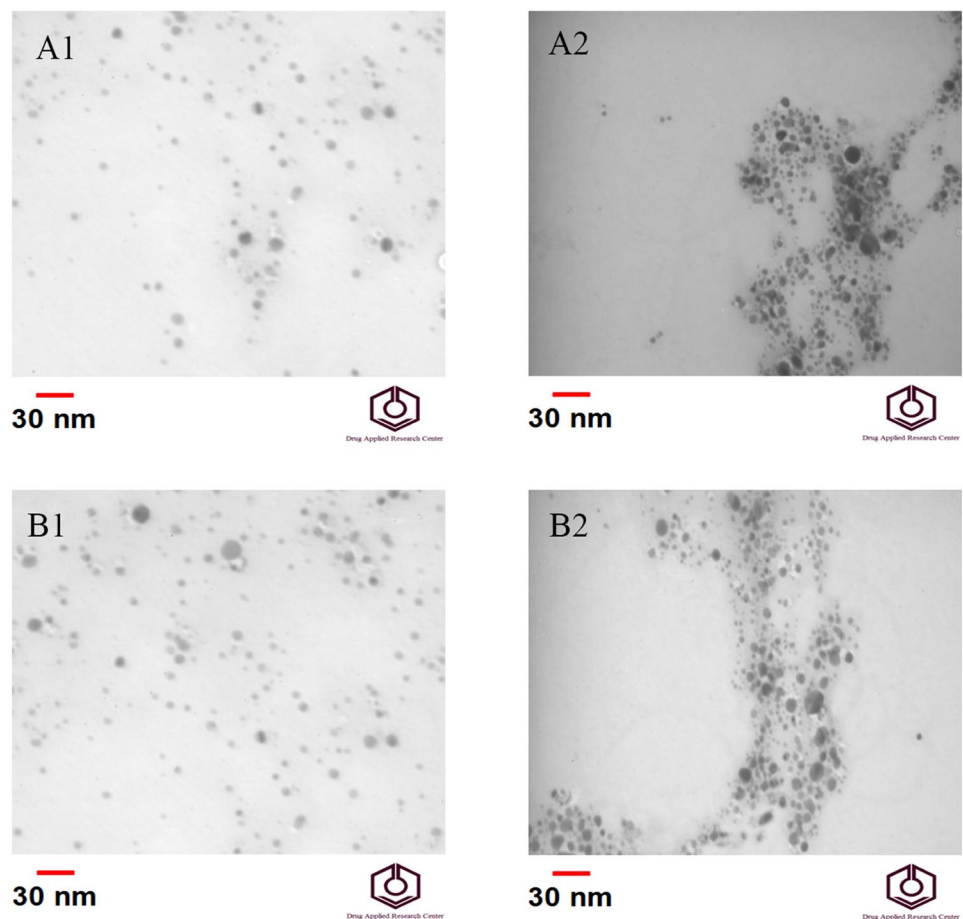

then tested to obtain a linear range with the best regression coefficient.

Then, ten different creatinine concentrations in the obtained linear range were tested to appraise the evaluation indices [such as limit of detection (LOD), standard deviation (SD), and relative standard deviation (RSD)]. It is important to note that in each experiment, three samples

with three different creatinine concentrations were selected and tested seven times to calculate the LOD.

As shown in Fig. 4, the linear range and limit of detection (LOD) were obtained for AgNPs/PVP as 0.03–1 mg/dl and 0.024 mg/dl and for AgNPs/PVA as 0.01–1 mg/dl and 0.014 mg/dl, respectively. The detectable resolution with AgNPs/PVP is 40 and with AgNPs/PVA is 71 for measuring

Fig. 3 TEM images from the samples; **a1** AgNPs/PVP in the absence of creatinine; **a2** AgNPs/PVP in the presence of creatinine; **b1** AgNPs/PVA in the absence of creatinine; **b2** AgNPs/PVA in the presence of creatinine; scale bar: 30 nm. According to the figure, after adding creatinine to the solution containing nanoparticles, it causes aggregation and discoloration in the solution

creatinine concentrations. Detectable resolution is the result of linear range's division by LOD. This index indicates the number of measurable points in each method. The colorimetric-based method proposed by Sergeyeva et al. (Sergeyeva et al. 2013) has a limit of detection of 2.83 mg/dl and a linear range of 2.83–28.3. Moreover, the detectable resolution of this method is 9. In addition, the linear range and LOD for the proposed method based on the Jaffé reaction combined with microfluidics technology introduced by Tseng (Tseng et al. 2018) were 0.19–7.64 mg/dl and 0.19 mg/dl, respectively. Furthermore, and the detectable resolution for this method was 37, indicating the superiority of the proposed method.
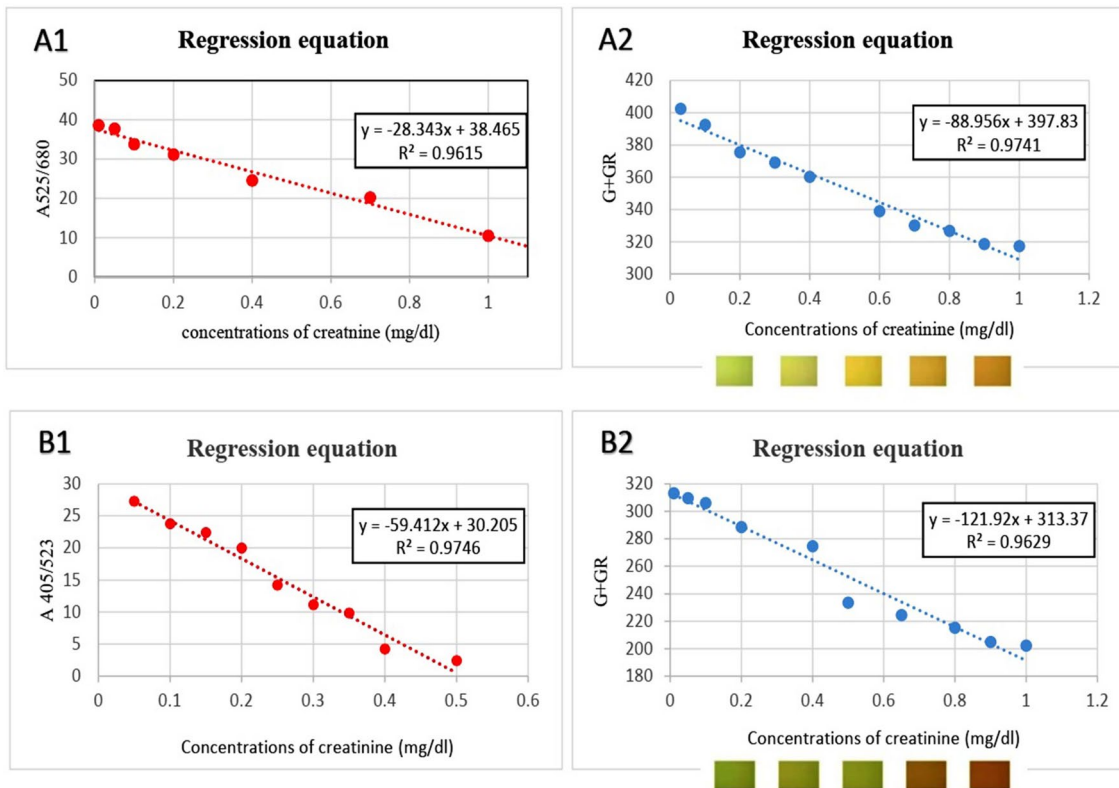
Discussion

Recently, the colorimetric method with image-processing technique has been used to measure the concentration of various compounds present in the human body. Therefore, the color changes in solutions containing AgNPs@PVP and AgNPs@PVA caused by different concentrations of creatinine are discussed in the current study. In this study,

this color change is measured using a designed optical imaging and image-processing system (colorimetric), and the amount of creatinine (quantitatively and qualitatively) is displayed in real time. For colorimetric processing, a variety of color systems, such as RGB (red, green, blue), CMYK (cyan, magenta, yellow, black), and grayscale (Gr), have been evaluated, indicating that the combination of green (G) and grayscale (Gr) provides the best results in this experiment. Possible noise in images is also eliminated by the software.

To compare selectivity, an experiment was performed using a number of compounds in blood and urine (interfering compounds). Solutions containing uric acid, glutamic acid, urea, arginine, cysteine, leucine, tryptophan, tyrosine, and glucose were used for this experiment. The results of this experiment are shown in Fig. 5. As can be observed, changes in color in different solutions in the presence of creatinine are negligible.

To analyze pH sensitivity, different pH ranges were created for each sample. Sodium hydroxide (NaOH) and hydrogen chloride (HCl) were used to adjust the pH of the solution. As shown in Fig. 6, the optimized pH for AgNPs@PVP is 6.5, while it is 7.5 for AgNPs@PVA.

C	A1	B1	A2	B2
Linear range(mg/dL)	0.01 - 1	0.05-0.5	0.03 - 1	0.01 - 1
LOD(mg/dL)	0.08	0.0375	0.024	0.014
Detectable resolution	12.3	12	40.42	70.72
SD(mg/dL)	0.01	0.0098	0.009	0.006
RSD%	11	9	8.79	7.96
R²	0.9615	0.9746	0.9741	0.9629

Fig. 4 Regression equations for **a1** spectroscopic analysis (A525/680) of AgNPs/PVP/creatinine; **a2** colorimetric (image processing) of AgNPs/PVP/creatinine; **b1** spectroscopic analysis (A405/523) of AgNPs/PVA/creatinine; **b2** colorimetric of AgNPs/PVA/creatinine. **c** Results obtained from data analysis. <<G = green Gr = grayscale >> // << A 405/523 = absorption ratio at 405–523>>

Moreover, the synthesized AgNPs@PVP and AgNPs@PVA nanoparticles are stable for more than 6 months. To demonstrate the stability of the synthesized nanoparticles, we prepared images and spectra of nanoparticles during a 6-month period. It should be noted that the synthesized samples were kept in an amber glass bottle at a temperature of 4 °C during this period. Figure 7 shows the results with regard to storage stability.

A table was compiled to compare the proposed method with other similar methods. As can be seen, this method,

while simple, provides acceptable and reliable results for measuring creatinine levels. The results of this comparison are presented in Table 1.

In terms of complexity and expensive instruments, this method is simple to use and requires no special training. Moreover, the cost of testing is much lower than that of other creatinine measurement methods (e.g., spectroscopy, chromatography, enzymatic, and so on). In addition, the proposed device can be used as a portable one.

Fig. 5 Colorimetric testing for system selectivity in the presence of interfering compounds; left: for AgNPs/PVP; right: for AgNPs/PVA; In the presence of 50 μM of each compound. The presence of creatinine in the solution, causing discoloration and decrease the amount of G+Gr
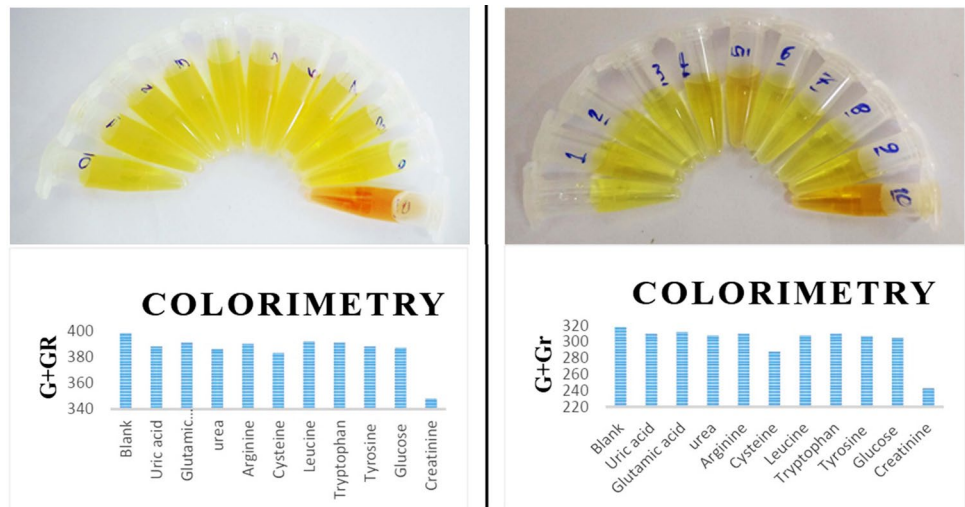

Fig. 6 PH sensitivity results for **a** AgNPs/PVP/creatinine and **b** AgNPs/PVA/creatinine. In **a** the solution (NPs) aggregates for pH greater than 10, before adding creatinine. In other pH except 6.5 for AgNPs@PVP and 7.5 for AgNPs@PVA), the effect of adding creatinine to the sample is negligible
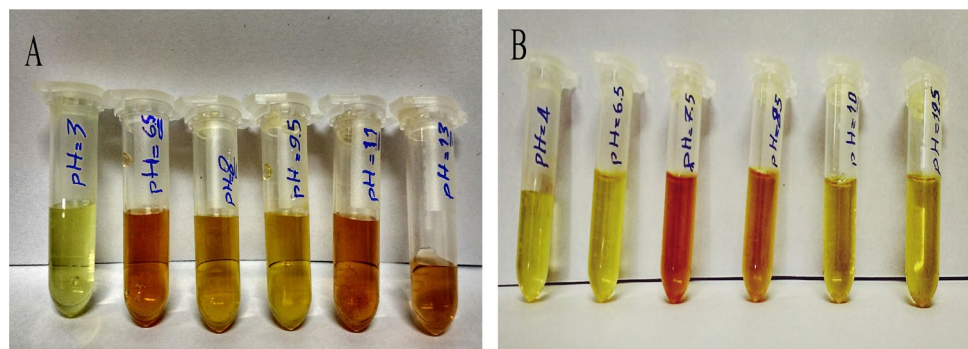

Fig. 7 Storage stability of the synthesized nanoparticles: left:AgNPs/PVP; right:AgNPs/PVA. The synthesized samples were kept in an amber glass bottle at a temperature of 4° C during this period
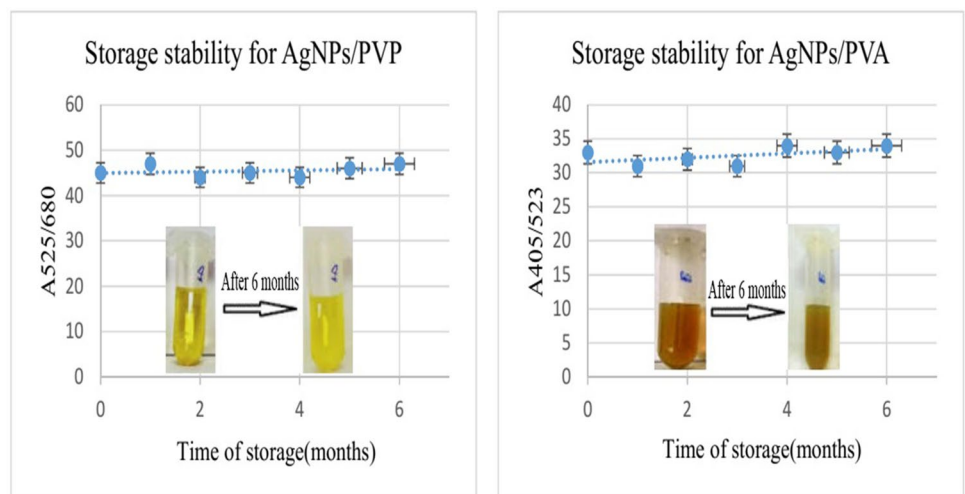
Table 1 Comparison of measurement indices and parameters of different methods with the presented method

References	Method		Linear range	LOD	Detectable resolution	Accuracy[a], %	SD, (mg/dl)	RSD, %	R^2	Storage stability
Sergeyeva et al. (2013)	MIP		2.83–28.3 mg/dl	2.83 mg/dl	10	–	–	–	–	1 year
Du et al. (2016)	UV–vis spectrometer		2.26–15.83 µg/dl	0.15 µg/dl	90	–	–	8	0.994	–
Alula et al. (2018)	Colorimetric		0–47.5 µg/dl	0.8 µg/dl	59	–	–	–	0.996	–
Tseng et al. (2018)	Microfluidic paper-based platform		0.19–7.64 mg/dl	0.19 mg/dl	39	–	–	–	0.99	100 days
Sittiwong and Unob (2015)	Spectrophotometer		1.5–4 mg/dl	1.37 mg/dl	3	–	1.37	9.7	0.95	–
This work	Colorimetric optical image processing	AgNPs/PVP	0.03–1 mg/dl	0.024 mg/dl	40	4.6	0.009	8.79	0.9741	6 month
		AgNPs/PVA	0.01–1 mg/dl	0.014 mg/dl	71	3	0.006	7.96	0.9629	6 month

[a] Accuracy% = (actual value−measured value)/(actual value)×100; the measured values are reported on average

Compliance with ethical standards

Conflict of interest We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

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