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ORIGINAL PAPER

Paper-based plasmon-enhanced protein sensing by controlled nucleation of silver nanoparticles on cellulose

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Abstract Cheap, disposable bio-diagnostic devices are becoming increasingly prevalent in the field of biosensing. Earlier we had reported the ability of cellulosic surface to control the nucleation of plasmonic silver nanoparticles and in this report we utilize this nucleation controlling property to demonstrate a new plasmonic sensing mechanism based on paper substrates to quantitatively detect proteins. On contrary to conventional paper based diagnostic devices which use the cellulosic part of paper as a support structure, the proposed method takes advantage of cellulose as nucleation controller during silver nanoparticle formation. Reduction of silver ions interacting competitively with nucleation controlling cellulosic surface and reduction suppressing amino acids of protein (via complexation) resulted in silver nanoparticles whose size–shape dependent plasmonic property quantitatively reflected the concentration of protein on paper, characterized using UV–Vis and surface-enhanced Raman spectroscopies. As a proof-of-concept, bovine serum albumin (BSA) was tested as the target analyte. UV–Vis spectroscopy based BSA quantification was sensitive in the concentration range 10–60 mg ml^{-1} while that for surface enhanced Raman spectroscopy extended well below 10 mg ml^{-1}, thus demonstrating the potential of this simple method to quantitatively detect a wide range of proteins relevant to the field of biodiagnostics.

Keywords Paper-biosensor · Cellulose-biosensor · Plasmonic-sensor · Biodiagnostics

Abbreviations

BSA	Bovine serum albumin
PTAP	4-Aminothiophenol
SERS	Surface enhanced Raman spectroscopy
UV–Vis	Ultra violet–visible light
XPS	X-ray photoelectron spectroscopy

Introduction

Interest in cellulosic fibers to develop advanced materials is exemplified by recent reports involving damage detection devices (Wandowski et al. 2011), electro-active paper (Kim et al. 2010), haptic sensors in prosthetic limbs (Yun et al. 2010), hydrophobic–lipophobic dirt-resistant coatings (Jin et al. 2011), piezoelectric materials (Lee et al. 2009), wireless communication devices (Kim et al. 2008), and

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biosensors (Martinez et al. 2010). This latter case is emblematic given the inherent low cost of cellulose-based sensors combined with a high performance, which have resulted in a wide range of commercially available biomedical tests and assays (Martinez et al. 2010; Rozand 2014). Paper is a network of cellulosic fibers assembled as a layered, random structure, which is well suited for functionalization with molecules or particles for enzymatic, optical or electrochemical response. Thus, paper has been extensively reported as ideal substrate for bio-diagnostic devices that take advantage of chemiluminescence, quantum dot luminescence, electrochemical, surface enhanced Raman spectroscopy (SERS) and enzymatic signaling for detection of glucose (Yu et al. 2011), catechols (Yuan et al. 2012), heavy metal ions (Nie et al. 2010), cancer cells (Liu et al. 2014) and immunogenic antigens (ELISA) (Cheng et al. 2010), respectively. In all paper-based sensing applications, the cellulosic fibers act as passive support to facilitate the interaction between analyte molecules and the sensing system, followed by transduction of a detectable signal. However, to our knowledge, no report exists about the use of cellulose as active component in paper-based sensing. Here we demonstrate a novel paper plasmonic sensing mechanism that directly and actively utilizes the characteristic surface of cellulose fibers.

Recently we have demonstrated the ability of cellulose to act as nucleation controller during silver nanoparticle formation whereby its surface was responsible for the size-dependent plasmonic properties of the nanoparticles (Lokanathan et al. 2014). Cellulose, as a polysaccharide, was demonstrated to be directly responsible for controlling the nucleation of silver nanoparticles, while surface anionic charges were responsible for stabilizing the nanoparticles (Lokanathan et al. 2014). Beyond its role as a mere passive support, we hypothesize that the nucleation controlling ability of cellulose could enable a sensing mechanism that is the result of its involvement in silver nanoparticle nucleation and growth. Paper mainly consists of cellulose, which according to our previous report (Lokanathan et al. 2014), controls the nucleation phenomena; such effect can be translated to its role in the form of a fiber network or paper. Hence it should be possible to develop a sensor based on silver nanoparticle nucleation by utilizing the polyol nature of fiber surfaces in paper. This hypothesis stems from the fact that reduction of silver ions on cellulose is highly sensitive to the interaction between the ions and the surface groups of cellulose. Therefore, any analyte molecule capable of disrupting or competing with this interaction is expected to affect the nucleation process in silver nanoparticle formation. Moreover, interference in silver nanoparticle nucleation may affect size- and shape-dependent plasmonic properties (Lokanathan et al. 2014). The relative shift in plasmonic signal of silver nanoparticles as a function of analyte concentration can be quantified by spectrometric measurements, for example, by UV–Vis absorption or SERS.

Here we demonstrate a cellulose-based sensing principle by using paper as active cellulosic substrate and BSA as a model analyte protein. This method is in contrast to silver staining procedures, for example, after gel electrophoresis of proteins (Merril 1990), where silver ion reduction proceeds in an uncontrolled fashion and results in micro-scale silver particles with no or negligible plasmonic properties. In our case the chemical characteristics of cellulose in paper controls the silver nanoparticle nucleation process thereby enabling the formation of plasmonic silver nanoparticles, thus acting as a quantitative signal rather than just a spatial staining-based indicator. Furthermore, the proposed paper-based plasmonic sensor exploits the nanoparticle formation step itself whereas in all other previously reported plasmonic sensors, the sensing step involves preformed nanoparticles (Lee et al. 2011; Liu et al. 2014). The quantitative sensing potential of the proposed system was demonstrated using bovine serum albumin (BSA) protein in the concentration range 1–60 mg ml^{-1}. The choice of the upper limit for the concentration range was based on the physiological concentration range of serum albumin in humans (HSA 30–50 mg ml^{-1}) (Choi et al. 2004). Overall, a simple plasmonic sensing mechanism is proposed that avoids the prerequisite of plasmonic nanoparticles of uniform size and shape and exploits the nucleation controlling ability of cellulose.

Materials and methods

Materials

Whatman cellulose chromatography paper (grade 2), silver nitrate ($AgNO_3$) (≥99.0), BSA (≥98 %, lyophilized powder) were purchased from Sigma-Aldrich. Milli-Q water (MQ, resistivity 18.2 MΩ) used for all solution making purposes was dispensed

by Millipore Synergy UV system. All chemicals and materials except BSA were used as received, without further purification. BSA was purified by dialysis against MQ water using a dialysis membrane until all the excess inorganic ions were removed. Spectra/por dialysis membrane (MWCO 500–1000) was purchased from Spectrum Laboratories Inc., Rancho Dominguez, California.

Procedure

The sequence of steps involved in the novel plasmonic paper-based protein sensing procedure is presented in Fig. 1. Chromatography paper (suitable for optical measurements) was used as source of cellulosic fibers. First, a 30 µl drop of solution of purified BSA (dialyzed against Milli-Q water to remove excess inorganic salts) was deposited on paper and allowed to dry under ambient conditions for 6 h. Subsequently a 30 µl drop of aqueous silver nitrate solution (6 mM) was deposited on the same location where BSA was deposited and allowed to dry for 15 h in darkness (ambient temperature and pressure). The concentration of BSA was varied between 0 and 60 mg ml^{-1}. Subsequently, the surface was exposed to UV light (λ-265 nm, power-5 mW cm^{-2}) for 0.5 h to reduce silver ions into metallic silver or silver nanoparticles. Note: there is the possibility for variations in results with the time allowed before measurement; however, this issue was not addressed here.

Before the addition of BSA solution (1st step in procedure) 30 µl water was deposited onto chromatography paper approximately at the same location where all subsequent additions were carried out. The added water was allowed to dry for 3 h before further steps were carried out. This pretreatment involving addition of water helped to ensure an homogeneous spreading of the components added subsequently, thus avoiding formation of undesirable rings of darker plasmonic nanoparticles, which can compromise the reproducibility of the measurement. The dialysis of BSA was very important due to the fact that certain inorganic anions such as chlorides form water-insoluble silver salts and may interfere the formation of plasmonic silver nanoparticles.

X-ray photoelectron spectroscopy

XPS survey and high resolution spectra were recorded using a Kratos Axis UltraDLD instrument (Kratos Ltd, Telford, UK) equipped with a monochromated aluminum anode (Al Ka 1486 eV) operating at 100 W power (12.5 kV and 8 mA) with 160 and 20 eV pass energies, respectively. The photoelectron take-off angle with respect to the surface's normal was 0° in all measurements. Charge correction for measured binding energies was performed with reference to 285.0 eV, corresponding to the C–C/C–H species. CasaXPS program was used to calculate relative atomic percentages of various samples from their respective survey spectra.

UV–Vis spectroscopy

UV–Vis diffuse reflectance spectra of sample surfaces were recorded using a PerkinElmer Lambda 950 UV/Vis/NIR absorption spectrophotometer. The measurements were performed within 24 h of UV exposure step in the sensing procedure described earlier.

Surface enhanced Raman spectroscopy

Ethanolic solution of 4-aminothiophenol (PTAP, 0.1 mM) was added onto all paper samples exposed to UV after addition of BSA and silver nitrate, respectively. All Raman spectra were acquired at ambient conditions using an alpha 300RA Combined Confocal Raman and AFM microscope system

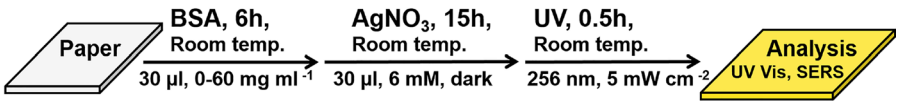

Fig. 1 Schematic illustration depicting the procedure involved in the paper-based plasmonic sensor. A drop of aqueous BSA solution was casted onto chromatography paper followed by addition of silver nitrate solution. After overnight evaporation in *dark*, the paper surface was exposed to UV light, followed by analysis using UV–Vis absorption and SERS. All steps in the procedure were performed at room temperature

(WITec, Inc., Ulm, Germany), using the 532 nm excitation of a neodymium doped yttrium aluminum garnet (Nd:YAG) laser and a 20× objective lens.

Results and discussion

Surface concentration of nitrogen and silver

X-ray photoelectron spectroscopy (XPS) was used to characterize the surface composition of paper after addition of BSA and silver nitrate, followed by UV treatment. The amount of BSA on the surface was quantified from the relative atomic percentage of N (Fig. 2a), which directly correlates with the surface concentration of BSA. Concentrations of BSA > 10 mg ml^{-1} showed no significant change in XPS's N %, thus it can be safely assumed that beyond this concentration the top 10 nm layer of paper surface was saturated with BSA molecules. Any excess BSA molecules would wick deeper into the paper network, making them undetectable due to limited analysis depth of XPS ($\sim$10 nm). In such a case, the excess BSA does not contribute to the nitrogen signal measured using XPS. In other words, once the solution of BSA comes in contact with top 10 nm of surface of paper, the BSA molecules start adsorbing onto the paper surface and upon saturation, the excess protein molecules left in the solution after saturation might spread deeper or wider across the cellulose fiber network through capillary effect. This surface saturation driven retention of BSA molecules on the top 10 nm of paper surface accompanied by steadily increasing penetration of BSA molecules, which could be responsible for the N % levelling off beyond 10 mg ml^{-1} BSA concentration. This speculation needs to be supported further by complementary characterization studies. Besides following nitrogen concentration, we also tracked the surface concentration of silver as a function of increasing BSA concentration (Fig. 2b). The amount of silver increased with increasing BSA concentration up to a BSA concentration of 2.5 mg ml^{-1}, beyond which point surface concentration (relative atomic percentages) of silver did not change significantly. The increased surface concentration of silver with increasing BSA concentration indicates a stronger interaction between silver ions and BSA when compared to interaction between silver ions and cellulose. It is well

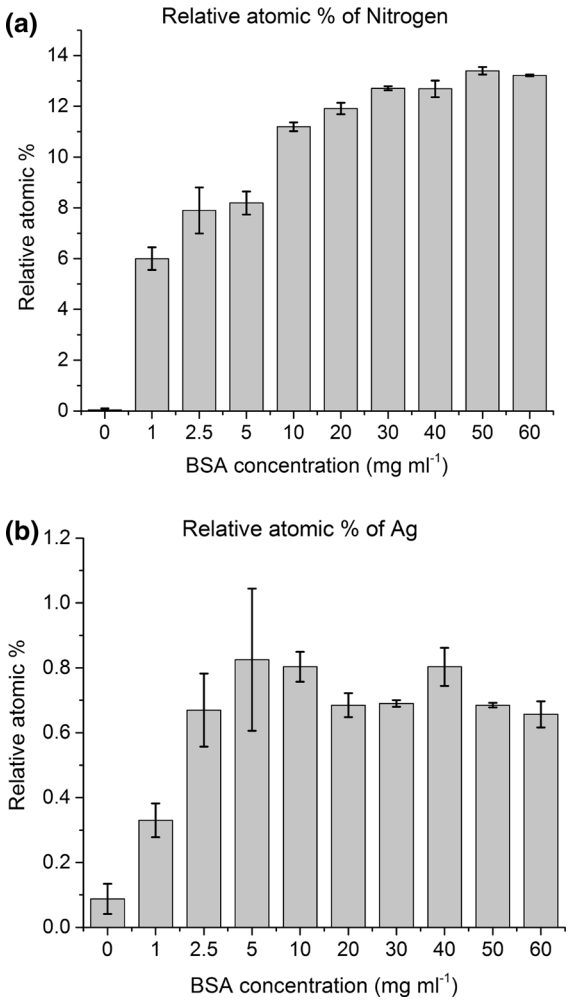

Fig. 2 XPS results. Relative atomic percentages of nitrogen (**a**) and silver (**b**) on the surface of paper exposed to UV light after addition of BSA and silver nitrate, respectively (calculation was based on the survey spectra). The concentration of BSA solution was varied from 0 to 60 mg ml^{-1}, while silver nitrate concentration was kept constant

known that proteins are capable of complexing silver ions (Merril 1990) and in our case it can be suggested that with increasing surface protein (BSA) concentration, an increased amount of silver ions are complexed. Based on this direct correlation between silver ion concentration and BSA surface content, it can be concluded that the silver ions interact strongly with BSA in the vicinity of the paper surface. It is known that strong complexation interactions affect the reduction potential of silver ions and thus significantly suppress the readiness with which they undergo reduction, which forms the basis of silver staining

protocols used in biotechnology (Merril 1990). BSA adsorbed Paper surface presents two contrasting types of interfaces to the incoming silver ions: nucleation controlling cellulosic surface; reduction suppressing BSA molecules. Thus, silver ion reduction induced plasmonic nanoparticles formation after UV exposure is expected to quantitatively reflect the competition between these contrasting interfacial phenomena and this forms the basis for a new sensing mechanism.

Visual analysis of effect of BSA on formation of silver nanoparticles

Photographs of various paper samples exposed to UV light after addition of BSA and silver nitrate, respectively are presented in Fig. 3. Plasmonic silver nanoparticles are responsible for the yellow color observed in these images and the changes in brightness and shade are related to the characteristic nanoparticle dimensions and number density (nanoparticles per unit area) (Morones and Frey 2007). It is very clear from Fig. 3 that with increasing BSA concentration the intensity of yellow color diminishes, thus indicating a corresponding decrease in plasmonic nanoparticle number density. Obviously, this decrease in plasmonic absorption based yellowness with increasing BSA concentration is due the increase in complexed silver ions (complexation by amino acids) resulting in decline of reduction capable silver ions, thus leading to decrease in the number of plasmonic silver nanoparticles formed after UV exposure. Finally, we note that SEM micrographs were recorded for all samples (not included here); no significant quantitative differences were noted in the presence or absence of BSA.

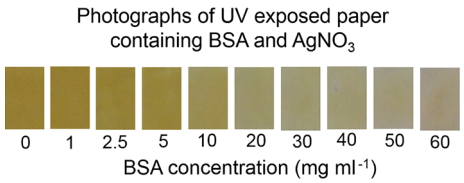

Fig. 3 Color photographs of various paper samples exposed to UV after addition of BSA and silver nitrate, respectively. The pictures are arranged in the order of increasing BSA concentration from *left* to *right* (0–60 mg ml^{-1}), as indicated. The silver nitrate concentration was kept constant in all samples

UV–Vis absorption studies

Besides visual analysis, the samples were also characterized using UV–Vis reflection absorption spectroscopy and the corresponding absorption spectra are presented in Fig. 4a. The spectroscopic studies indicate that the intensity of extinction peak decreases with increased BSA concentration beyond 10 mg ml^{-1} and the extent of intensity reduction becomes quantitatively obvious from Fig. 4b where the extinction value at λ_{MAX} of plasmon peak is plotted as function of BSA concentration. A direct relationship between intensity at λ_{MAX} and BSA concentration in the range 10–60 mg ml^{-1} is observed, thus demonstrating the potential sensing ability of paper-based plasmonics to quantify human serum albumin whose physiological concentration varies between 35 and 50 mg ml^{-1} (Choi et al. 2004). UV–Vis absorption seems to have a lower limit of 10 mg ml^{-1} for quantitative analysis of the model protein but the combined use of SERS extends the potential of paper-based plasmonic sensing to detect even lower protein concentrations. The rationale behind using SERS shall be explained next followed by results of SERS studies.

SERS studies

Results of UV–Vis absorption studies presented earlier in this report indicate that the number density of plasmonic silver nanoparticles decreased with increasing BSA concentration. Thus, it would be expected that the Raman signal would scale inversely with BSA concentration when equal amounts of a Raman active molecule, capable of high surface enhancement, is added onto the paper samples containing varying number densities of silver nanoparticles. To test this hypothesis we performed experiments using 4-aminothiophenol (PTAP, 10^{-4} M), a Raman active molecule with good surface enhancement characteristics with silver nanostructures (Zheng et al. 2003).

Raman spectra of PTAP added plasmonic paper samples with varying amounts of BSA are presented in Fig. 5a. The intensity of surface enhanced Raman peaks corresponding to PTAP was observed to decrease with increasing concentration of BSA and this decrease becomes quantitatively apparent from the plot of Raman peak intensity at 1372 cm^{-1} as a

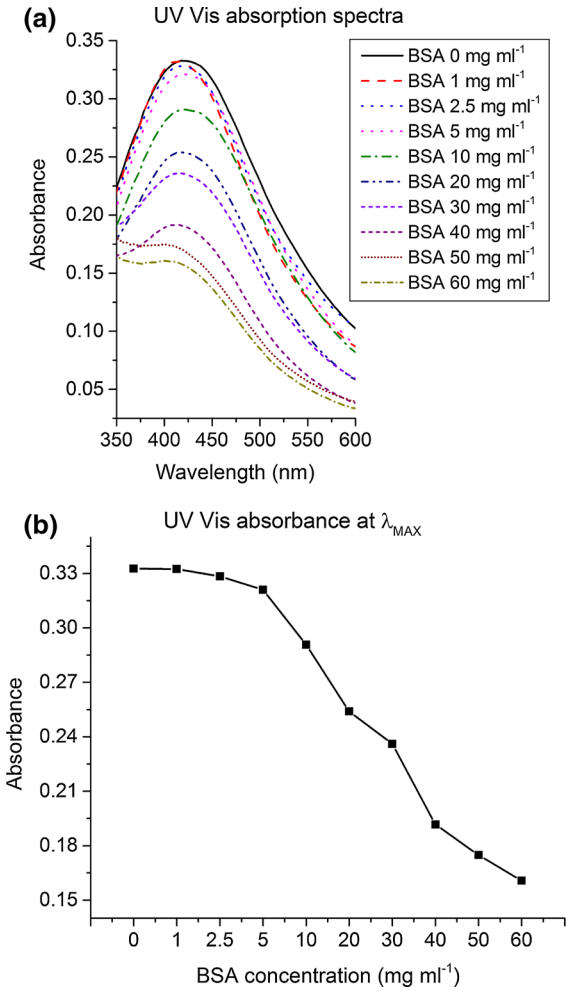

Fig. 4 UV–Vis absorption spectra (**a**) and plasmon peak intensity at λ_{MAX} as a function of BSA concentration (**b**) measured for various paper samples exposed to UV after addition of BSA and silver nitrate, respectively. Silver nitrate concentration was kept constant in all samples. The absorption spectrum of chromatography paper was subtracted from that of the paper samples

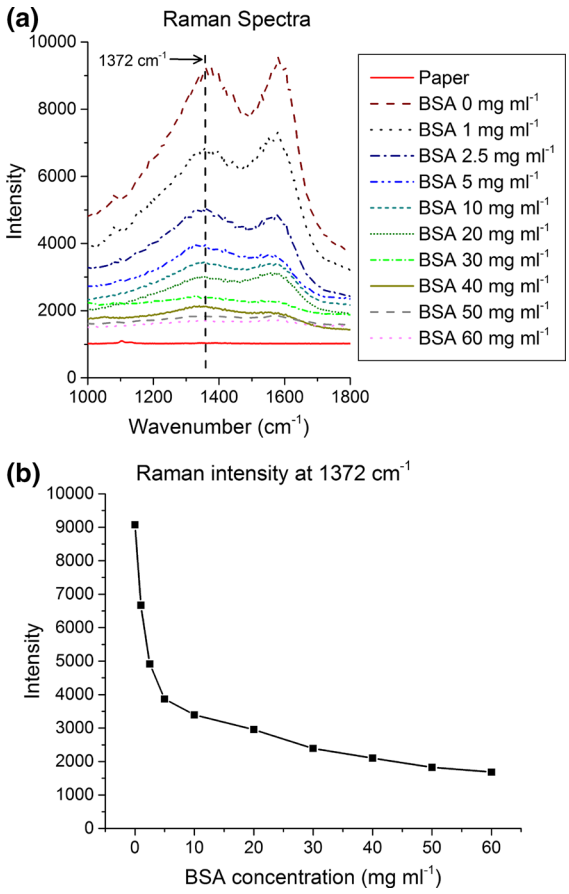

Fig. 5 Raman spectra of plasmonic paper samples with added PTAP (**a**) and Raman peak intensity at 1372 cm^{-1} as a function of BSA concentration (**b**) measured for various paper samples exposed to UV after addition of BSA and silver nitrate, respectively. The concentration of silver nitrate and PTAP were kept constant in all samples, except for sample labeled 'Paper'

function of BSA concentration, as shown in Fig. 5b. Between 0 and 10 mg ml^{-1}, the Raman intensity decreases by 60 %, on the other hand subsequent increments of 10 mg ml^{-1} BSA concentrations resulted in an average intensity decrease of 13 ± 4 %, thus indicating that SERS based sensing procedure is quantitatively more sensitive to the model protein in the concentration range 0–10 mg ml^{-1}.

Discussion

Overall we present a novel paper-based sensing method that uses the effects of plasmonic nanoparticle nucleation at cellulosic interfaces. The idea is that the number of silver nanoparticles decrease with increasing concentration of BSA. The correlation between the concentration of BSA and number density of silver nanoparticle was quantified via UV–Vis and Raman spectroscopy. A linear relationship was observed for BSA concentrations in the range 0–10 and 10–60 mg ml^{-1} for Raman (SERS) and UV–Vis

spectroscopies, respectively. Though the work presented here uses BSA as a model protein, this mechanism is expected to be applicable to other analytes, including proteins, DNA, heavy metal ions, etc., all of which competitively interfere with the silver ion–cellulose interactions. There is a need for deployment in real applications by challenging the systems with protein mixtures. At this stage our efforts provide a proof of concept to open the proposed approach for further studies toward the development of cheap, paper-based biosensors.

We foresee that the proposed paper plasmonic sensor, which gives quantitative information about the concentration can be extended to paper electrophoresis, where separation of protein molecules, and thus the position of the plasmonic stain would indicate the identity of the protein (based on electrophoretic mobility), while the plasmon based spectroscopy would enable quantification. Further investigations are ongoing to improve the sensitivity and extend the application of this novel mechanism to detect a broader range of analytes. It is worth mentioning that utilizing nanocellulose instead of micro-scale fibers (paper) would in-crease the specific-surface area of substrate and consequently may increase the detection limit and sensitivity.

Conclusions

As a follow up to our earlier report demonstrating the ability of cellulosic surface to control the nucleation of silver nanoparticles, we demonstrate a paper based plasmonic sensing mechanism using BSA as a model analyte. The surface concentration of protein and silver on paper, quantified using XPS indicated a direct quantitative relationship between the two elements, which was attributed to the ability of protein molecules to complex silver ions. The protein's ability to complex silver ions was responsible for the diminished formation of plasmonic nanoparticle with increasing BSA concentration due to hindrance in the nucleation of silver nanoparticles at cellulosic interface. This formed the basis for an inverse quantitative relationship between the concentration of protein and surface plasmonics dependent optical property quantified using analytical techniques including UV–Vis absorption and SERS. When applied to this plasmonic paper, the SERS technique was found to be capable of extending the detection limit below 10 mg ml^{-1}. In contrast, using UV–Vis absorption technique enabled the quantitative detection in the 10–60 mg ml^{-1} concentration range. Overall we demonstrate the potential applicability of a novel sensing mechanism which directly utilizes the cellulosic surface of paper which is in contrast to conventional paper based sensing devices using the cellulosic part of paper as passive support for the sensing molecules.

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