Bioplasmonic paper–based assay for perilipin-2 non-invasively detects renal cancer

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Renal cell carcinoma (RCC) has poor survival prognosis because it is asymptomatic at an early, more curative stage. Recently, urine perilipin-2 (PLIN-2) was demonstrated to be a sensitive and specific biomarker for the noninvasive, early detection of RCC and an indispensable indicator to distinguish cancer from a benign renal mass. However, current Western blot or ELISA PLIN-2 assays are complicated, expensive, time-consuming or insensitive, making them unsuitable for routine analysis in clinical settings. Here we developed a plasmonic biosensor based on the high refractive index sensitivity of gold nanorattles for the rapid detection of PLIN-2 in patient urine. The paper-based plasmonic assay is highly sensitive and has a dynamic range of 50 pg/ml to 5 μg/ml PLIN-2. The assay is not compromised by variations in urine pH or high concentrations of interfering proteins such as albumin and hemoglobin, making it an excellent candidate for routine clinical applications. The urine PLIN-2 assay readily distinguished patients with pathologically proven clear cell carcinomas of various size, stage and grade (55.9 [39.5, 75.8] ng/ml, median [1st and 3rd quartile]) from age-matched controls (0.3 [0.3, 0.5] ng/ml), patients with bladder cancer (0.5 [0.4, 0.6] ng/ml) and patients with diabetic nephropathy (0.6 [0.4, 0.7] ng/ml). Urine PLIN-2 concentrations were roughly proportional to tumor size (Pearson coefficient 0.59). Thus, this cost-effective and label-free method represents a novel approach to conduct a non-invasive population screen or rapid differential diagnosis of imaged renal masses, significantly facilitating the early detection and diagnosis of RCC.

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Kidney cancer accounts for 3% to 4% of the newly found cases of malignant tumors in adults.[1] Unfortunately, renal cell carcinoma (RCC) is generally asymptomatic at early stages and is thereby frequently advanced when a diagnosis is made. Most renal masses are incidentally discovered by imaging modalities such as computed tomography and magnetic resonance imaging, but imaging cannot reliably distinguish between cancerous and benign tumors.[2,3] The conventional approach to a suspected renal mass is partial or radical nephrectomy. The problem is that 20% of imaged renal masses are not cancer, and patients incur the loss of normal kidney tissue,[2,3] reducing renal function and accelerating progression of underlying chronic kidney diseases.[3,4]

Recently, we reported that urine perilipin-2 (PLIN-2) and aquaporin-1 are sensitive and specific biomarkers for early noninvasive detection of clear cell or papillary subtypes of RCC (together accounting for 85%–90% of malignant RCC).[5,6] These biomarkers were not increased in patients with common noncancerous kidney diseases (i.e., diabetic nephropathy, glomerulonephritis, urinary tract infection[5]) or other urinary tract cancers (bladder or prostate[6]), thus demonstrating specificity. Results suggested that aquaporin-1 and PLIN-2 have potential utility for differential diagnosis of imaged renal masses.[5,6] However, the Western blot procedure for PLIN-2 is complicated, expensive, is semiquantitative, and time-consuming, which is not suitable for analysis of urine samples in clinical lab settings. It is also challenging to develop an enzyme-linked immunosorbent assay for PLIN-2, particularly for screening of patient urine samples, due to insufficient sensitivity and effects of interindividual variability in urine pH. A convenient and cost-effective assay is needed for fast and quantitative testing of urine PLIN-2. This could significantly facilitate differential diagnosis of imaged renal masses to avoid unnecessary renal excision surgery and its consequences.

Plasmonic biosensors, based on reflective index sensitivity of localized surface plasmon resonance (LSPR), are highly sensitive, cost-effective, and label-free biodiagnostic platforms. LSPR of noble metal nanostructures involving the

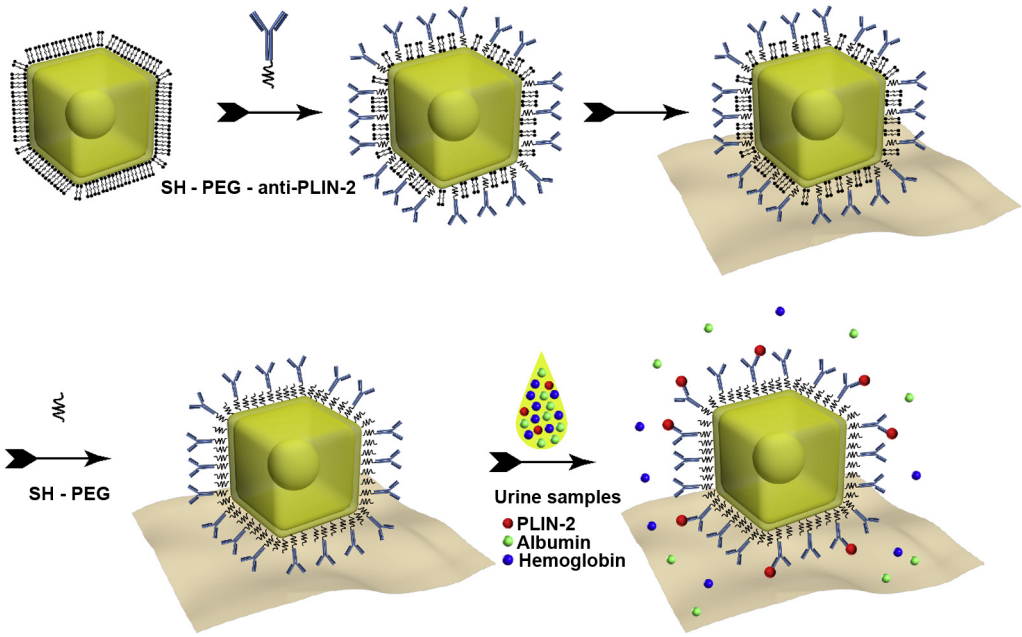

Figure 1 | Schematic illustration of plasmonic paper manufacture. Gold nanorattles (AuNRTs) coated with cetyltrimethylammonium chloride were functionalized with monoclonal antibody by means of a bifunctional polyethylene glycol (PEG). The functionalized AuNRTs were then coated on filter paper, and nonspecific binding sites were neutralized by further PEGylation. The paper-bound functionalized AuNRTs then bind specific analytes, in this case, perilipin-2 (PLIN-2), and cause a red shift in the localized surface plasmon resonance for the AuNRTs. SH, a thiolated polyetheylene glycoll.

coherent oscillation of dielectrically confined conduction electrons is a sensitive methodology.[7–9] Hollow plasmonic nanostructures such as gold nanoshells, nanocages, and nanorattles exhibit significantly higher refractive index sensitivity than do their solid counterparts, making them excellent candidates for plasmonic biosensors.[10] Recently, we have demonstrated that gold nanorattles (AuNRTs) exhibit much higher refractive index sensitivity than do gold nanorods with similar LSPR wavelength.[11]

Plasmonic paper, common filter paper adsorbed with biofunctionalized nanostructures, is a highly attractive biodiagnostic platform for point-of-care and resource-limited settings.[12–16] Our previous work has provided proof-of-concept for bioplasmonic paper as a platform technology for the detection and quantification of aquaporin-1, another biomarker for RCC diagnosis.[17]

Herein, we report a facile approach for rapid and highly sensitive detection of PLIN-2 in clinical urine samples. Common filter paper uniformly absorbed with commercial monoclonal antibody-conjugated AuNRTs is employed as a label-free plasmonic biosensor. This assay is significantly more sensitive than Western blotting and provides the requisite sensitivity for quantifying clinical urine PLIN-2 concentrations for RCC. Moreover, this technology represents a highly attractive diagnostic platform for rapid detection and quantification of target bioanalytes in preclinical and clinical settings.

RESULTS

A schematic illustration summarizing the building of the bioplasmonic paper to detect PLIN-2 using AuNRTs and a monoclonal antibody is depicted in Figure 1. The technology is versatile with the assay specificity dependent on the specificity of the antibody, and the sensitivity of the assay is amplified by the nanoparticle. The paper base allows easy handling and processing.

Measurement of PLIN-2 in human urine

The remarkable reproducibility of the bioplasmonic paper assay for PLIN-2 is illustrated in Supplementary Figure S1, which shows that 2 years apart the standard curves are indistinguishable. The LSPR wavelength shift of the bioplasmonic paper on exposure to kidney cancer patient urine samples was found to be significantly higher compared with that of healthy volunteers (example in Figure 2a). Results were also compared with PLIN-2 concentrations previously determined by Western blot analysis.[5,6] Median urine PLIN-2 concentrations from the 20 patients with RCC were 43 (interquartile range [IQR]: 29, 68) ng/ml and were significantly higher ($P < 0.001$, Kruskal-Wallis) than those of 20 controls at 0.3 (IQR: 0.3, 0.5) ng/ml, those of 8 patients with bladder cancer at 0.5 (IQR: 0.4, 0.6) ng/ml, or those of 10 patients with diabetic nephropathy at 0.6 (IQR: 0.4, 0.7) ng/ml (Figure 2b). There was no overlap in PLIN-2 concentration ranges between RCC and all other groups. Equally important, the urine PLIN-2 concentrations were related to tumor size (Figure 2c) with a Spearman correlation coefficient of 0.59 (95% confidence interval: 0.16 to 0.81; $P < 0.009$). A Spearman plot of the urine PLIN-2 concentrations of the patients with RCC measured by the plasmonic papers and previously by Western blotting (Figure 2d) indicates an

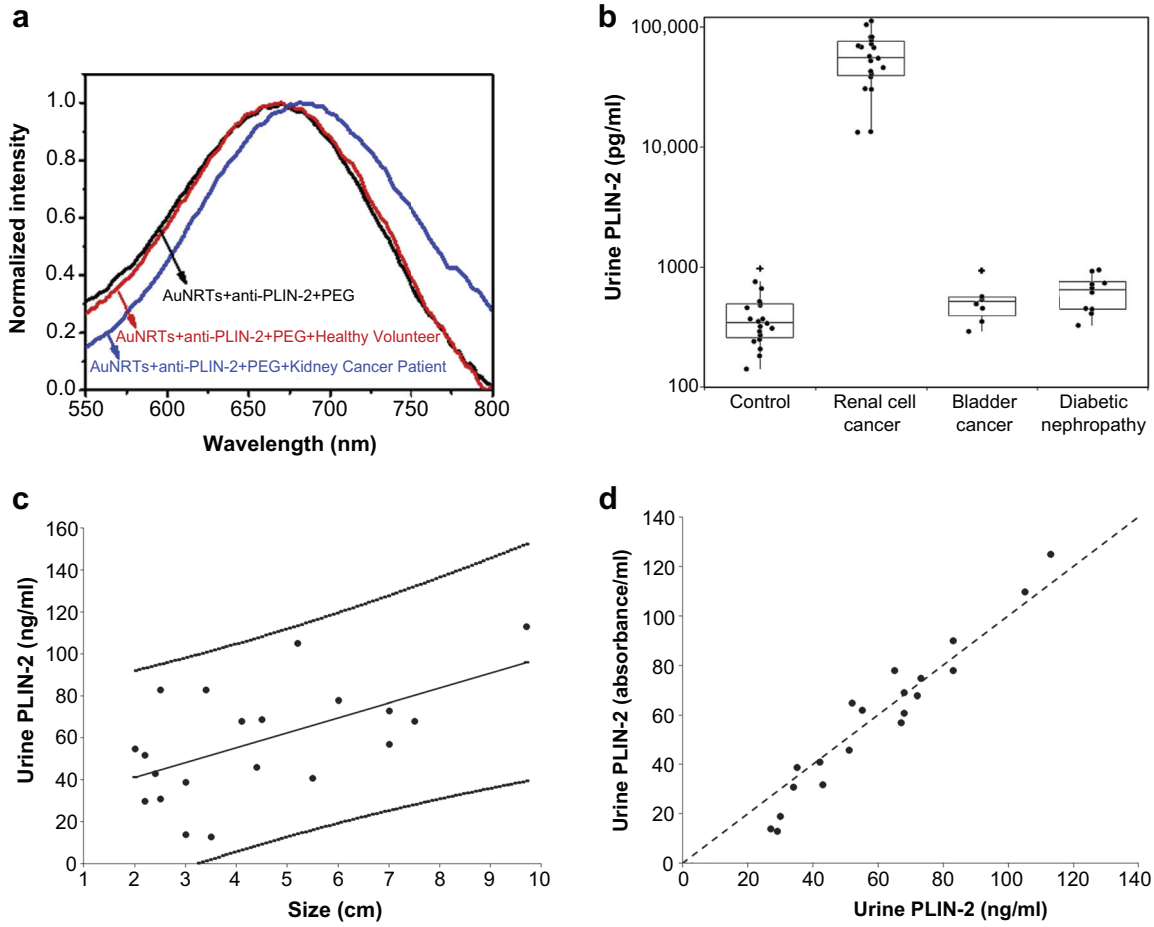

Figure 2 | (a) Representative localized surface plasmon resonance spectra of anti–perilipin-2 (anti-PLIN-2) functionalized gold nanorattles (AuNRTs) (black), functionalized AuNRTs incubated with urine from a healthy volunteer (red), and functionalized AuNRTs incubated with urine from a patient with renal clear cell carcinoma (blue). (**b**) Box and whisker plot of the urine PLIN-2 concentrations of 20 control patients, 20 patients with renal cell cancer, 8 patients with bladder cancer, and 10 patients with diabetic nephropathy. The median with whiskers and boxes depicting the interquartile range is shown. The concentration of each patient is depicted as a solid dot or a plus sign. The plus signs are outliers more than 1.5 times but less than 3.0 times the interquartile range. Patients with renal cell cancer have significantly higher urine PLIN-2 concentrations than that of other cohorts ($P < 0.001$ by Kruskal-Wallis). (**c**) Urine PLIN-2 concentration of patients with renal cell cancer as a function of tumor size. The Spearman correlation coefficient is 0.59 ($P = 0.009$). Regression line, solid line; 95% prediction interval, dashed lines. (**d**) Urine PLIN-2 concentration determined by plasmonic paper versus relative absorbance by Western blot. Dashed line of identity. Spearman correlation = 0.94 ($P < 0.001$). PEG, polyethylene glycol.

excellent correlation ($R^2 = 0.94$; $P < 0.001$). However, it should be noted that the Western blot analysis appears to tail off at low PLIN-2 concentrations.

DISCUSSION

Plasmonic paper serves as a simple and versatile platform for implementing plasmonic biosensors. Due to the high sensitivity of the assay, and lack of potential interference by albuminuria or hematuria, urine PLIN-2 concentrations in normal individuals could be accurately measured, and there was a clear difference in urine PLIN-2 concentrations among normal individuals, patients with bladder cancer, patients with diabetic nephropathy, and patients with a pathologically proven clear cell carcinoma. A limitation of the study is the small number of patients in this proof-of-concept analysis. A larger multicenter blinded study incorporating patients with a wide variety of noncancerous kidney diseases, more patients with cancerous urologic diseases, and many more control individuals would be necessary to validate this assay. The assay enables fast, sensitive, cost-effective, and non-invasive diagnosis of kidney cancer, especially in at-risk groups. For example, the Veterans Health Administration now targets kidney cancer as a specific condition based on the exposure of soldiers, their families, and base personnel to contaminated drinking water.[18] There is also a significantly increased incidence of RCC among autoworkers exposed to metal-working fluids.[19] There are no current cost-effective high-throughput means of identifying individuals with RCC. Our assay could be of importance in identifying those with RCC in military bases or autoworker cohorts or other populations. Additionally, this assay would enable the differential diagnosis of imaged renal masses and could be useful in point-of-care settings such as hospital radiology departments and stand-alone imaging clinics. The need for differential diagnosis of

imaged renal mass is compelling. Nationally, about 45,000 partial and radical nephrectomies were performed in the United States alone in 2015.[3] It is estimated that 18% of these would have undergone surgery for removal of a benign mass (extrapolation of the Surveillance, Epidemiology, and End Results [SEER] 18 database). A non-invasive biomarker assay to differentiate a benign mass from a renal cell cancer could potentially have prevented unnecessary surgery in these cases. Future prospective studies are needed to validate this bioplasmonic paper–based assay in population screening and to differentially diagnose imaged renal masses on a larger scale.

METHODS

Synthesis of bioplasmonic paper

We employed a seed-mediated approach in combination with galvanic replacement reaction to synthesize AuNRTs.[11] These AuNRTs were conjugated with a commercially available human PLIN-2–specific monoclonal antibody as the biorecognition element. The successful conjugation of the PLIN-2 antibody was evidenced by a 6-nm red shift in the LSPR wavelength of the AuNRT (Supplementary Figure S2B). Uniform adsorption of bioconjugated plasmonic nanostructures on the paper substrate, which ensures optical homogeneity of the plasmonic paper substrates, is of great importance in determining the limit and accuracy of detection. Scanning electron microscopy images revealed that the AuNRTs were uniformly distributed on paper substrates without significant aggregation or patchiness (Supplementary Figure S2C). Furthermore, surface-enhanced Raman scattering spectra confirm successful conjugation of anti-PLIN-2 IgG_{2B} to the AuNRTs (Supplementary Figure 2D). Characteristic Raman spectral bands at 905 cm^{-1}, 960 cm^{-1}, 1233 cm^{-1}, and 1307 cm^{-1} (correspond, respectively, to symmetric C-C-N stretching and CH_3 rocking vibration and asymmetric C-C-N stretching of the IgG_{2B}) confirm the successful biofunctionalization of the nanostructures with antibodies.

Measurement of PLIN-2 in patient urine

Urine from 20 patients with documented clear cell renal carcinomas, 8 patients with documented bladder cancer, and 20 normal individuals were obtained after written informed consent from September 2013 through February 2014. Approval was acquired from the Washington University Institutional Review Board. The studies were registered with ClinicalTrials.gov (NCT00851994 and NCT01538823). Urine from patients with diabetic nephropathy was originally obtained from the P30 (DK079333) Kidney Translational Research Core of the Washington University George M. O'Brien Center for Kidney Disease Research as part of a previous study.[20] Characterization of the patient cohorts is presented in Figure 2a.

The concentration of PLIN-2 was measured from October to November 2016, again in November 2017, and in March 2019 in a blinded fashion by immersing the plasmonic paper substrates into the different calibration standards or patient urine (diluted 10-fold) for 2 hours, after which they were completely rinsed with water and dried under a stream of nitrogen. Six visible to near–infra-red extinction spectra were obtained for each paper substrate using a spectrophotometer coupled to an optical microscope before and after being exposed to samples to ensure uniformity of the readings. The average LSPR wavelength before incubation of the paper substrates was subtracted from the average wavelength after incubation (Figure 2a), and the PLIN-2 concentration was calculated based on the standard curve run within each assay.

Details of the synthesis and characterization of the bioplasmonic papers, patient demographics, and statistical methods used are presented in the Supplementary Information.

DISCLOSURES

JJM, SS, and EDK are among founding members of Auragent Bioscience, LLC, which is a small business startup developing plasmonic amplification of fluorescent signals, and, to date, has no financial interest in LSPR-based assays. All the other authors declare no competing interests.

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PLIN-2, also known as ADFP, is subject in part to patents US9091690, US9494590, EP2433137, and EP2863225 to EDK and JJM and patent US9410949B2 to EDK, JJM, and SS. To date, there are no financial interests related to these patents.

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SUPPLEMENTARY MATERIAL

Supplementary File (Word)

Supplementary Information about assay reproducibility, quality control, and tumor characteristics.

Figure S1. PLIN-2 standard curves 2 years apart.

Figure S2. (**A**) Characterization for the gold nanorattles (AuNRTs). (**B**) LSPR shift after anti-PLIN-2 IgG bound to AuNRTs. (**C**) Density of the AuNRTs on the bioplasmonic paper. (**D**) Raman shifts characterizing IgG bound to the AuNRTs.

Figure S3. Composite curve of 5 separate PLIN-2 standard curves (mean LSPR shifts with SDs).

Figure S4. Dilution of PLIN-2 standard in synthetic urine or pooled normal human urine.

Figure S5. (**A,B**) Histogram of AuNRTs LSPR peak reproducibility.

Figure S6. Homogeneity of LSPR spectra across the plasmonic patch.

Figure S7. (**A,B**) Specificity of the plasmonic patches and stability to urine pH.

Figure S8. PLIN-2 assay quality control.

Table S1. Assay reproducibility.

Table S2. Deming regression analysis: significant *P* values.

Table S3. Assay quality control.

Table S4. RCC tumor stage and grade.

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