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Nanoplasmonic Sensing from the Human Vision Perspective

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ABSTRACT: Localized surface plasmon resonance (LSPR) constitutes a versatile technique for biodetection exploiting the sensitivity of plasmonic nanostructures to small changes in refractive index. The optical shift in the LSPR band caused by molecular interactions in the vicinity of the nanostructures are typically < 5 nm and can readily be detected using a spectrophotometer. Widespread use of LSPR based sensors require cost effective devices and would benefit from sensing schemes that enables use of very simple spectrophotometers or even naked eye detection. This contribution describes a new strategy facilitating visualization of minute optical responses in nanoplasmonic bioassays by taking into account the physiology of human color vision. We demonstrate, using a set of nine different plasmonic nanoparticles, that the cyan to green transition zone at ~500 nm is optimal for naked eye detection of color changes. In this wavelength range it is possible to detect a color change corresponding to a wavelength shift of ~2-3 nm induced by refractive index changes in the medium or by molecular binding to the surface of the nanoparticles. This strategy can also be utilized to improve the performance of aggregation-based nanoplasmonic colorimetric assays, which enables semi-quantitative naked eye detection of matrix metalloproteinase 7 (MMP7) activity at concentrations that are at least 5 times lower than previously reported assays using spherical gold nanoparticles. We foresee significant potential of this strategy in medical diagnostic and environmental monitoring, especially in situations where basic laboratory infrastructure is sparse or even non-existent. Finally we demonstrate that the developed concept can be used in combination with cell phone technology and RGB analysis for sensitive and quantitative detection of MMP7.

Refractometric or refractive index (RI) biosensors based on LSPR are widely used for biointeraction analysis in research and development and has a large number of potential applications in medical diagnostics, food and water quality control and environmental monitoring.¹ LSPR benefit from a very high surface sensitivity and low temperature dependency, and require less complicated optical components than most other optical biosensor techniques. However, despite extensive optimization and development of various intricate plasmonic nanostructures, such as nanorods,² cubic boxes,³ mushrooms⁴, nanorings⁵, nanohole arrays,⁶ nanotriangles⁷, nanodisks,⁸ mesoflowers,⁹ coupled nanoparticles¹⁰, alloy nanoparticles,¹¹ nanoparticle polymer composite¹² and nanoparticles of other non-noble metals^{11, 13} semiconductors¹⁴ and dielectric materials,¹⁵ detection of the optical shift in the LSPR band caused by small RI changes require a spectrophotometer. This is contrast to another aspect of nanoplasmonic sensing exploiting changes in colloidal stability triggered by analyte recognition. Aggregation or redispersion of plasmonic nanoparticles tend to produce massive shifts in the LSPR band that easily can be detected by the naked eye. Naked eye detection is of large interest for bioanalytical applications targeting situations where basic laboratory infrastructure is sparse or even non-existent, such as in assays for medical diagnostic or environmental monitoring in developing countries. Reducing the need for, or complexity of, additional equipment required for sensor readout is also motivated from a cost perspective in home-based or point-of-care diagnostics.

Mirkin et. al. pioneered the development of colorimetric bioassays for polynucleotides utilizing controlled aggregation of gold nanoparticles (AuNPs) in response to hybridization of

complementary oligonucleotide strands.¹⁶ Aggregation of the AuNPs resulted in a significant red shift of the LSPR band and in a distinct color change of the AuNP suspension/spot from red to blue. Exploiting similar strategies, numerous assays for detection of target molecules with diverse physical and chemical properties since then have been reported including DNAs,¹⁶⁻¹⁷ proteins^{17a, 18} small biological molecules,¹⁹ metal ions,²⁰ enzymes,^{2, 9, 21} toxins,²² gas molecules²³ etc. Most often, colorimetric responses in the high target concentration regime are readily distinguishable by the human eye, while those in the low concentration regime still require the use of a spectrometer. Therefore, methods that could tune the responses of LSPR RI sensors to enable naked eye detection would thus be of high relevance as this could facilitate development of very cost-effective biosensor solutions and open up for a much wider range of both applications and technology end users.

In this contribution, we investigate a strategy to use LSPR to detect small RI changes by naked eye without further improving the RI sensitivity of the nanostructures, but rather focusing on the physiological aspects of human vision. The LSPR band of plasmonic nanoparticles red shifts both with an increase of the local RI and as a result of aggregation of the nanoparticles.²⁴ Efforts to improve the RI sensitivity of LSPR have typically resulted in nanoparticles with more initially red shifted LSPR bands. In fact, there is linear relationship between the RI sensitivity and the position of the LSPR band.²⁵ Ironically, as the LSPR band is pushed into the red region of the spectrum, the possibilities for human eye detection is diminished due to the physiology of color vision.

Figure 1. (A) Color spectrum in the visible range. The positions corresponding to the spectra in B and C are indicated with black circles. (B) Normalized extinction spectra and photos of plasmonic nanoparticle with a shift of 9.7 nm starting from ~ 500 nm. (C) Normalized extinction spectra and photos of plasmonic nanoparticle with a shift of 14.4 nm starting from ~ 660 nm.

Three types of cone cells are responsible for our perception of color, namely short (S), medium (M) and long (L) cone types; each stimulated by overlapping spectral bands with distinct peak wavelength regimes at 420-440 nm for S, 534-555 nm for M and 564-580 nm for L.²⁶ Due to this color sensitivity they are also denoted as blue, green and red cones, respectively. The human photopic eyes show the highest sensitivity in the green spectral range, according to eye sensitivity function.²⁷ Moreover, the distribution of colors is far from uniform as shown by the color bands of the visible spectrum from 400 nm to 700 nm (Figure 1A). For example, the cyan and green color bands are much narrower than the red color band. For this reason, the same amount of shift starting from different positions in the visible range yields varying degrees of color change. Thus, in order to maximize the resulting perceived color change the starting (baseline) position of the LSPR band, prior to exposure of the sample, should fulfill the following criteria: 1) appear within a narrow spectral range/color band; 2) produce a distinct and sharp color transition in response to the sample and 3) appear in the cyan-green range. Taking these criteria into account, we identified the cyan to green transition zone centered ~ 500 nm, (left circle in Figure 1A), to be the most favorable position to visualize spectral changes caused by red shift in the LSPR band.

To illustrate the impact of the starting/baseline position of the LSPR band on the perceived color change, two plasmonic nanoparticles with different LSPR extinction maxima (500 nm and 660 nm, Figure 1B-C) are compared. A plasmonic nanoparticle sample that absorbs strongly in the visible range of the spectrum are perceived as complementary colors. Note, the extinction of nanoparticles is defined as the sum of absorption and scattering of the light. For the set of NPs studied here, except for the nanoshell and nanostar NPs, the scattering is relatively low meaning that extinction $\approx$ absorption. The color we see (perceive) after letting white/broad band light (Figure 1A) pass a suspension of NPs that absorbs light in a particular region of the visible spectrum is the complementary color to the absorption or LSPR band of the NP.” For instance, the complementary colors of cyan/green correspond to orange/brown. Figure 1B shows the photos and the corresponding extinction spectra of plasmonic nanoparticles with a LSPR band at ~ 500 nm where a RI-induced optical shift $\Delta\lambda \sim 9.7$

nm results in a distinct change of the complementary colors that is readily visualized. On the contrary, for plasmonic nanoparticles with a more redshifted LSPR band (Figure 1C), a triggered LSPR shift of the same magnitude ($\Delta\lambda \sim 14.4$ nm) produce a color change that is very difficult to discern because of the broadness of the red band and consequently the small difference in complementary colors (dark green). From this comparison, it is clear that to improve the visual perception sensitivity of a color change, the position from which the optical shift starts is far more critical than the magnitude of the optical shift $\Delta\lambda$. Translating this observation into the language of materials design - the plasmonic nanoparticles that possess a LSPR excitation maximum in the sharp cyan/green regime are better suited for naked eye detection than those with resonances in the red part of the spectrum, despite their higher $\Delta\lambda$ /RI sensitivity of the latter.

We conducted a systematic study to examine the influence of the starting position of LSPR band on the perceived color changes in response to minute RI changes. We show that, by using nanoparticles with extinction bands near the cyan to green transition zone ~ 500 nm, color changes corresponding to minute wavelength shifts $\Delta\lambda \sim 2-3$ nm induced by RI changes are readily distinguishable. We also show that this strategy is applicable for improving the sensitivity in conventional aggregation based colorimetric nanoplasmonic assays. We anticipate that the proposed human vision oriented strategy can provide a new concept for designing generic colorimetric sensors that can enable rapid and sensitive detection of molecular interactions, either by naked eye for semi-quantitative analysis or by using very simple ubiquitous, technologies, such as a cell phone camera, for the development of portable and cost effective quantitative bioassays.

Experimental Section

Materials

Neutravidin was purchased from Thermo-Scientific. Peptide 1 with the sequence CALNN and peptide 2 with the sequence CALNN(PEG)₄SNKTRIDEANQRATKLLGSGDDGDDGDD were purchased from GL Shanghai, China. AuNPs of size 40nm, 60nm and 80nm were purchased from BBI solutions. Hydrogen peroxide 30%wt was purchased from VWR chemicals, Poland. Gold (III) chloride trihydrate (99.9%), sodium citrate tribasic dehydrate (99%), ascorbic acid (99%), silver nitrate (99%), cetyl trimethylammonium bromide (96%) (CTAB), chitosan (low molecular weight), acetic acid (99.7%), 3-Mercaptopropionic acid (99%) (MPA), hydroxylamine hydrochloride (99%), sodium borohydride (98%), sodium chloride (99%) and glycerol (99%) were purchased from Sigma Aldrich. Hydrochloric acid (37%) was purchased from Merck Millipore, Singapore. The synthesis of the polypeptide JR2EC with the sequence (NAADLEKAIEALEKHLEAKGPCDAAQLEKQLEQAFEA FERAG) has been described elsewhere.²⁸ MMP7 human recombinant, E. Coli was purchased from Merck Chemicals.

Synthesis and characterization of nanostructures

AuNPs with a diameter of 20 nm were prepared by citrate reduction of HAuCl₄. Briefly, a volume of 102 mL MilliQ water was added into a round bottom flask and stirred, followed by adding 12mL HAuCl₄ (2.54 mM) into the round bottom flask and heating it to boil under a condenser. Then 6 mL of 10 mg/mL sodium citrate was injected and boiling was continued until the solution turned to red color (about 30 min).

Au/Ag alloy nanoparticles of different compositions were synthesized by simultaneous reduction of the mixture of HAuCl_4 and AgNO_3 in trisodium citrate solution.²⁹ The composition of Au and Ag was tuned by the fraction of HAuCl_4 and AgNO_3 . Take $\text{Au}_{0.7}\text{Ag}_{0.3}$ NP as an example, 875 μL of HAuCl_4 (10 mM) aqueous solution was added into 50 mL Milli-Q water with refluxing. Then 375 μL of AgNO_3 (10 mM) aqueous solution was added into the reflux solution, which was kept stirring for 30 min to obtain a uniform mixture of gold and silver ions. After that, 2.5 mL of trisodium citrate aqueous solution (1wt%) was quickly added into the mixture and the color of the solution gradually changed to orange. The solution was refluxed for another 30 min, and then cooled down to room temperature. The volume of 10 mM HAuCl_4 and 10 mM AgNO_3 for $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP are 960 μL and 240 μL , respectively. The volume of 10 mM HAuCl_4 and 10 mM AgNO_3 for $\text{Au}_{0.6}\text{Ag}_{0.4}$ NP are 720 μL and 480 μL , respectively. Au Shells were synthesized by galvanic replacement of the core Ag nanoparticles with Au ions following the recipe described elsewhere.³⁰ Au Star was synthesized following the recipe described elsewhere.³⁰

UV-Vis spectra of all nanoparticles were obtained using a UV-Vis spectrometer (PerkinElmer Lambda 35). The morphology and size of all nanoparticles were characterized by transmission electron microscope (TEM) with JEOL 2010 operating at 200 kV. The composition of gold/silver alloy particles were studied by energy-dispersive X-ray spectroscopy (EDX) with JEOL-7600) working at 15 kV.

Refractive index and molecular binding measurement

Solvents with refractive index from 1.33 to 1.42 were prepared by changing the fraction of glycerol from 0 to 60% in water. Nanoparticles were dissolved in these solvents and absorption spectra/images were taken in cuvette. Adsorption of MPA, peptides and neutravidin onto the $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs, 20 nm AuNPs and 60 nm AuNPs were conducted for 100 μM of the molecules dissolved in 10 mM sodium citrate buffer at pH 6 to prevent aggregation of the nanoparticles.

Survey on color change

A small cohort of 44 persons with different ethnic backgrounds, age, gender and occupations were asked to evaluate the color changes of the photos shown in Figure 4A. The subjects participating in the survey include researchers of Chinese, Indian, European and Korean origin in NTU, service staff in NTU and professional workers in industries outside NTU. Another survey was made on the response of AuNPs and $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs with different concentrations of MMP7, Figure 6B and 6C. For this survey, a group of 10 people were asked to give opinion on the color changes. People who participated in the survey were not aware of the purpose and this study. They were asked to rank the photos with respect to the most distinct color changes. The counts for the four best samples were tabulated and the score of each sample was calculated. The scores were obtained by calculating the sum of weighted counts, for more details see Table S1 in the supplementary information.

MMP7 assay

Both the AuNPs of 20 nm size and $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs were functionalized with a 42-mer peptide (JR2EC) by incubating the NPs in 10 μM JR2EC solution for 8 hr. The nanoparticles (2mL) were then washed in PBS buffer pH 7.4 followed by centrifugation and removal the supernatant. Four repeated

Figure 2. (A) Normalized extinction spectra of NPs 1-9 and (B) TEM images of the plasmonic NPs. 1. $\text{Au}_{0.6}\text{Ag}_{0.4}$ NP, 2. $\text{Au}_{0.7}\text{Ag}_{0.3}$ NP, 3. $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP, 4. 20 nm AuNP, 5. 40 nm AuNP, 6. 60 nm AuNP, 7. 80 nm AuNP, 8. Au Shell NP and 9. Au Star NP. Scale bar: 20 nm.

washing steps were carried out to remove the free JR2EC in solution. At the end of the washing step, the nanoparticles were dispersed in 500 μL PBS buffer for future use. Sensing was done by using MMP7 stock solution of 2 mg/mL and 200 μL nanoparticles.

Cell phone and RGB analysis

All photos were taken using CCD camera and the cell phone based MMP7 sensing was done with a smartphone (Huawei P9) with a built-in camera (Leica Summarit H 1:2.2/27 ASPH). The RGB analysis of photos was done with the software ImageJ. First, the R, G and B components of a photo were calculated, and then the color perception (brightness) was calculated with the formula: $Y = 299R + 0.587G + 0.114B$.

Results and discussion

The resonance wavelength of plasmonic nanoparticles varies with their size, shape, and composition and is also influenced by the RI of their immediate vicinity.²⁴ For instance, spherical AuNPs with 20 nm diameter exhibit a LSPR band at ~ 520 nm. By increasing the size of the AuNPs to 80 nm the LSPR band shifts ~ 30 nm to the red. Other gold nanostruc-

tures, such as Au shells or Au stars, demonstrate an additional shift to the red. On the other hand, alloying spherical AuNPs with Ag atoms results in a shift of the LSPR band towards shorter wavelengths. Thus, by carefully controlling the ratio of Au and Ag, alloy nanoparticles with a LSPR band in the cyan to green transition zone ~ 500 nm can be prepared.

To examine the impact of the position of the LSPR band on the actual spectrophotometric and perceived color change of plasmonic nanoparticles when changing the RI, a library of plasmonic nanostructures covering a wide range resonance wavelengths across the visible spectrum from 400 to 700 nm were synthesized. Figure 2A and 2B show the normalized extinction spectra and TEM images of the synthesized nanoparticles. Nanoparticles with LSPR bands from 400 nm to 520 nm can be synthesized by varying the composition of Au and Ag (Figure S1). The composition of the three synthesized alloy nanoparticles²⁹ $\text{Au}_{0.6}\text{Ag}_{0.4}$ NP, $\text{Au}_{0.7}\text{Ag}_{0.3}$ NP and $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP (Figure 2, 1-3) were characterized with EDX (Figure S2). Nanoparticles with LSPR bands from 520 nm to 600 nm were obtained³¹ by varying the particle size, from 20-80 nm (Figure 2, 4-7). Nanoparticles with a LSPR band in the range from 600 nm to 700 nm were obtained by synthesizing Au Shell³² and Au Star³³ (Figure 2, 8-9). It is worthwhile noticing that among these nanostructures the alloy nanoparticle $\text{Au}_{0.8}\text{Ag}_{0.2}$ (NP 3, Figure 2) has a LSPR band in the cyan to green transition zone, i.e. ~ 500 nm, shown by the left circle in Figure 1A.

The LSPR band red shifts with increasing RI for all the nanoparticles used. However, the magnitude of the redshift differs. The extinction spectra of the nanoparticles shown in Figure 2 were measured in solvents with refractive indices increasing from 1.33 to 1.42 (Figure S3). The profiles of the shift as a function of refractive index for each nanoparticle are plotted and linearly fitted in Figure 3A. The change in extinction maximum as a function of refractive index for the nanoparticle suspensions are plotted in Figure S4. The change in intensity (extinction) with increasing refractive index follows essentially the same trend except for the nanoshell and nanostar NPs, see Figure S4A. The refractive index sensitivity for all the nanoparticles is summarized in Figure 3B. It reveals that Au Star demonstrates the highest RI sensitivity (235 nm/RIU), followed by Au Shell (227 nm/RIU, Figure 3B), which is in good agreement with previous findings suggesting that nanoparticles with more red-shifted LSPR band generally yield higher refractive index sensitivity.³⁴ Notably, the refractive index sensitivity of the $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP is 86 nm/RIU, which ranks it seventh out of the nine nanoparticles (Figure 3B).

To examine whether the visible color change of the nanoparticle is dictated by the refractive index sensitivity, we studied the color of the nanoparticle solutions with different refractive indices (1.33, 1.36, 1.39 and 1.42) by naked eye inspection. The photos of different nanoparticles in solvents possessing different refractive indices are shown in Figure 4A. Interestingly, the perceived color change correlates poorly to the actual spectral shift. Briefly, the degree of perceived color change for the nanoparticles in response to increasing refractive index could be categorized into two groups. The first group, which comprises the three alloy particles $\text{Au}_{0.6}\text{Ag}_{0.4}$ NP, $\text{Au}_{0.7}\text{Ag}_{0.3}$ NP and $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP, shows color changes that readily can be distinguished by the naked eye. The second

Figure 3 (A) Summary of peak shift of the plasmonic NPs vs refractive index. The peak shift is linearly fitted, where the 60 nm AuNP and $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs overlap. (B) The refractive index sensitivity of the NPs.

group, comprising the remaining nanoparticles does not produce a pronounced color change.

It is critical to note that the perceived color of an object is subjective due to slight difference in our visual system and thus varies from person to person. To study the variation in perception of color changes, we conducted a survey to evaluate the perception of the color changes of the 9 photos in Figure 4A. A small cohort of 44 individuals was asked to rank the four samples of nanoparticles with the highest degree of color change and the scores were calculated. More details of the survey method and data analysis can be found in the supplementary information and Table S1. The survey revealed that the majority of the votes/counts were given to alloy NPs. The $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP exhibited the highest count and score, followed by the $\text{Au}_{0.7}\text{Ag}_{0.3}$ NP and $\text{Au}_{0.6}\text{Ag}_{0.4}$ NP, respectively (Figure 4C). On the other hand, the Au Shell, Au Star, 40 nm AuNP and 20 nm AuNP (Figure 4B) received very low scores. The Au Star and Au Shell NPs, although displaying very good RI sensitivity, produced negligible color changes.

Figure 4 (A) Photos of synthesized plasmonic nanoparticles in solution with increasing RIs from left to right: 1.33, 1.36, 1.39 and 1.42, respectively. The peak shift that varies linearly with RI (see Fig. 3A) is also shown above the first and last photo in the sequence. (B) Counts given to the samples with the most obvious color change (rank 1st), see the first row in Table S1. (C) Score for each sample, see the last row in Table S1.

The superior visual color change of Au_{0.8}Ag_{0.2} NP and the discrepancy with respect to the UV-Vis is obvious. In fact the closer one can tune the nanoparticle composition to the transition regime the better visual color perception sensitivity. Thus, it is critical to carefully choose the starting wavelength of the LSPR band in order to properly visualize minute color changes. The LSPR band of Au_{0.8}Ag_{0.2} NP is located in the cyan to green transition zone ~500 nm, while the Au_{0.7}Ag_{0.3} NP and Au_{0.6}Ag_{0.4} NP has an offset of ~10 nm and ~20 nm towards shorter wavelengths. All other nanoparticles display LSPR band wavelengths beyond the cyan to green transition zone. Our findings demonstrate that Au_{0.8}Ag_{0.2} is the most suited for visualizing small optical shifts. Importantly, with the Au_{0.8}Ag_{0.2} NPs, one can distinguish, by naked eye, a color change that corresponds to a peak shift of 2-3 nm in the LSPR spectrum (the peak shift between the 1st and 2nd cuvette in Figure 4 is 2.5 nm). It is worthwhile to mention that although the perceived color change also may originate from changes in the intensity/extinction, we did not observe significant differences in the change of intensity for the Au and AuAg alloy

NPs with increasing RI. In fact the particle that showed the most prominent color change, the Au_{0.8}Ag_{0.2} NP, displayed a significantly smaller intensity change with RI as compared to Au NP of similar size for the same wavelength shift (5nm), Figure S4B. Moreover, the width of the LSPR band determines the perception of colors from bright colors (for sharp LSPR bands) to dull colors (for broad LSPR bands), see Figure S5. As the LSPR for the NPs investigated herein is relatively broad we do observe dull colors, e.g. brownish (Figure 1B) instead of bright orange as expected from the ideal case for sharper resonances.

The ability to visualize a color change corresponding to a LSPR band shift of a few nm opens up for using of Au_{0.8}Ag_{0.2} NPs to directly explore molecular adsorption/binding of potential target molecules to the nanoparticle surface. To mimic a sensing scenario and to test the performance of the Au_{0.8}Ag_{0.2} NPs in detecting molecular binding, a number of molecules with different molecular weights (from 105 Da to 60 kDa) were chemisorbed/physisorbed to the nanoparticle surface. The normalized extinction spectra of Au_{0.8}Ag_{0.2} NP modified

Figure 5 (A) Normalized extinction spectra of $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs modified with sodium citrate (black), MPA (red), Pep 1 (green), Pep 2 (blue) and neutravidin (pink). Note, the spectra of the blank, MPA and Pep 1 are overlapping. The band positions for solutions appear at 502.4, 502.1, 504.1, 506.9 and 509.1 nm, respectively. (B) Photos of $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP modified with different molecules from left to right: blank/sodium citrate, MPA, Pep 1, Pep 2 and N. Avidin, respectively.

with the different molecules are shown in Figure 5A. As expected, adsorption of these molecules induced minute redshifts of < 7 nm. The color changes for $\text{Au}_{0.8}\text{Ag}_{0.2}$ NP modified with Pep 2 and N. Avidin are readily distinguishable from the samples referred to as blank (sodium citrate capped NPs), Figure 5B. For the smaller molecules, MPA and Pep 1, the perceived color changes were not as pronounced although most of the individuals asked could distinguish between MPA and Pep 1. As a comparison, when these molecules were adsorbed to 20 nm AuNPs and 60 nm AuNPs no color changes could be detected for neither of them, Figure S6. It is worthwhile to notice that the change in RGB colors induced by molecular binding also can be recorded using a commercial software package, ImageJ. With RGB analysis a quantitative response can be obtained, Figure S7.

To further demonstrate the possibility to use this concept for improving the sensitivity of aggregation based nanoplasmonic colorimetric detection schemes, we revisited a previously developed assay for the detection of MMP7.⁴² Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling.³⁵ They also play a major role in cell behaviors such as cell proliferation, migration, differentiation, apoptosis, and host defense. Moreover, the MMPs are considered useful early biomarkers for many cancers due to the fact that they are heavily up-regulated during disease development.³⁶ Therefore, extensive research interests have been devoted to the development of sensors and assays,³⁷ imaging technologies³⁸, drug

Figure 6 (A) Illustration of colorimetric sensor for MMP7. (B) Photos of AuNPs and $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs with different concentrations of MMP7. Black line shows the threshold of detection for naked eyes. (C) Changes in ΔY ($Y = 299R + 0.587G + 0.114B$) of $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs (black) and AuNPs of 20 nm size (red) with concentration of MMP7, where the data for $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs and AuNPs are fitted exponentially. The dashed lines show the 3 times of the noise level of $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs (black) and AuNPs (red), respectively.

delivery and cancer therapies³⁹ and MMP responsive biomaterials.⁴⁰ The principle of the employed colorimetric assay is schematically illustrated in Figure 6A. Briefly, MMP7 digests the JR2EC-peptide immobilized on the $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs and the 20 nm AuNPs for comparison. This digestion reduces the electrostatic repulsion between the NPs resulting in a concentration-dependent aggregation of the NPs and concomitant change in the color of the NP suspension.²⁸ Figure 6B shows the photos of the 20 nm AuNPs and $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs with different concentrations of MMP7 from 0 to 5 $\mu\text{g/mL}$, respectively.

According to a survey of a small group of 10 observers, the color change for AuNPs with 2.5 $\mu\text{g/mL}$ is clearly seen, but the concentrations below that cannot be distinguished, Figure 6B. While for the $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs, the color change of 0.2

$\mu\text{g/mL}$ -0.5 $\mu\text{g/mL}$ is visible with the naked eyes. Thus, by simply changing the nanoparticles from AuNPs to $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs, the concentration distinguishable from the blank for naked eye detection of MMP7 can be lowered by at least a factor of 5, see solid line in Figure 6B. Again most of the individual in this cohort of observers could distinguish the 0.2 $\mu\text{g/mL}$ sample from those of lower concentrations (0 and 0.125 $\mu\text{g/mL}$).

As mentioned before, the present naked eye approach is ideal for rapid threshold semi quantitative type analysis. However, to enable quantitative and sensitive analysis of MMP7 and related cancer biomarkers at clinically relevant concentration levels we have to combine our assay with a more sensitive and objective readout technology. Again we have chosen to use the camera function of mobile phone and RGB analysis to turn the proposed colorimetric assay into an objective, quantitative and more sensitive tool.⁴¹ Mobile phone has many advantages as a diagnostic tool, e.g. build-in camera, communication, calculation and data storage function as well as its wide availability and friendly user interfaces.⁴² All these features make it a promising portable and affordable platform for applications in medical diagnostic, environmental monitoring, high throughput screening and for online applications in the process industry. We demonstrate herein a RGB analysis based on images taken by the camera of a mobile phone to study of colorimetric response of JR2EC-functionalized $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs and 20 nm AuNPs (as a reference) upon exposure to MMP7. Figure 6C shows the changes in RGB value (ΔY) of $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs and 20 nm AuNPs for different concentrations of MMP7. The ΔY values of $\text{Au}_{0.8}\text{Ag}_{0.2}$ and Au NPs change exponentially with the concentration of MMP7 in the range of 0 to 5 $\mu\text{g/mL}$. The LOD calculated using 3 time noise level equals 0.082 $\mu\text{g/mL}$ and 1.25 $\mu\text{g/mL}$ for the $\text{Au}_{0.8}\text{Ag}_{0.2}$ and Au NPs, respectively. Notably, the LOD using $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs is even better than the corresponding LOD obtained with a fine-tuned UV/Vis spectrometer for 20 nm AuNPs ~ 0.1 $\mu\text{g/mL}$.²⁸ Interestingly, Whitesides et. al. also demonstrated that the RGB analysis using the mobile phone shows comparable accuracy as commercial bench top spectrometers.⁴³ Furthermore, the current assay in conjunction RGB analysis potentially enables early diagnosis of cancers patients whose MMP7 levels typically fall in the range 0.1 - 2 $\mu\text{g/mL}$.²⁸ We therefore believe that the $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs can be used as a general plasmonic material to replace the AuNPs for the development of portable colorimetric sensors to improve the LOD. In addition, it is worthwhile mentioning that the response of this $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs based sensor is fast and a clear colorimetric response can be visualized in less than 30 seconds (see video in supporting information). Considering the superior sensitivity for human eye detection and fast response, we believe that alloy NPs will find many applications in environmental sensing, biomedical diagnostics and potentially also for high throughput screening applications.

Conclusions

We have reported a strategy to optimize performance of LSPR based RI nanoplasmonic assays from the human vision perspective. Due to the unique features of the human eye, the ability to detect minute color changes in response to refractive index changes in the surroundings critically depend on the choice of starting wavelength prior to exposure to the sample solution. With consideration to the vision mechanism and the property of visible spectrum, we identified the cyan to green

transition zone at ~ 500 nm to be the best starting wavelength for visualization of color changes. A number of different plasmonic nanoparticles with different LSPR band wavelengths were synthesized and evaluated for both optical spectral shift and color changes. We observed a huge discrepancy between optical shift and perceived color change, where the Au Star experienced a significant LSPR band shift (14.4 nm) but negligible color changes. On the contrary, the $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs which absorb at the cyan to green transition zone ~ 500 nm shows pronounced color changes. Importantly, using $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs, one could readily visualize the color change corresponding to minute LSPR band shifts of 2-3 nm induced by a refractive index change. Adsorption of biomolecules like peptides and proteins to $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs could also induce an obvious color change from orange to red. In addition, we employed a previously developed colorimetric assay for MMP7 to evaluate this concept to also enhance the performance of aggregation based nanoplasmonic biodetection. Using $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs the concentration for visual detection could be lowered by at least 5 times compared to that of 20 nm AuNPs. We also demonstrated that mobile phone technology and RGB analysis enabled rapid, sensitive and quantitative detection of MMP7 at clinically relevant levels. We believe that the proposed strategy could be easily and widely employed to improve current refractometric and colorimetric LSPR based sensors, but also enable development of new generic colorimetric sensors. This human vision-oriented strategy may also find applications in the analysis of other color related phenomena beyond sensing.

ASSOCIATED CONTENT

Supporting Information

Extinction peak position of Au/Ag alloy nanoparticles as a function of the composition of Au atoms, EDX characterization of Au/Ag alloy nanoparticles, normalized extinction spectra of all the plasmonic nanoparticles in solvents with different refractive indices, summary of survey results, photos of 20 nm AuNPs and 60 nm AuNPs with adsorption of molecules of different molecular weight, photos and video of $\text{Au}_{0.8}\text{Ag}_{0.2}$ NPs based sensor for MMP-7. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

No conflict interests are reported between authors.

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