Gold/silver/gold trilayer films on nanostructured polycarbonate substrates for direct and label-free nanoplasmonic biosensing

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Ultrasmooth gold/silver/gold trilayer nanostructured plasmonic sensors were obtained using commercial Blu-ray optical discs as nanoslits-based flexible polymer substrates. A thin gold film was used as an adhesion and nucleation layer to improve the chemical stability and reduce the surface roughness of the overlying silver film, without increasing ohmic plasmon losses. The structures were physically and optically characterized and compared with nanostructures of single gold layer. Ultrasmooth and chemically stable trilayer nanostructures with a surface roughness <0.5 nm were obtained following a simple and reproducible fabrication process. They showed a figure of merit (FOM) value up to 69.2 RIU−1 which is significantly higher (more than 95%) than the gold monolayer counterpart. Their potential for biosensing was demonstrated by employing the trilayer sensor for the direct and refractometric (label-free) detection of C-reactive protein (CRP) biomarker in undiluted urine achieving a Limit of Detection (LOD) in the pM order.

KEYWORDS
Blu-ray disc, flexible substrate, nanoplasmonic devices, nucleation layer, silver nanoslits

Surface plasmon resonance (SPR) phenomena in metallic nanostructures have undergone extensive development due to their ability to confine light at the nanoscale and the possibility of coupling and tuning different photonic effects (eg, Fabry-Perot cavity, Wood’s anomalies or Fano resonances). These characteristics confer on SPR the potential for widespread use...
in applications related to photon management [1], microscopy [2], surface-enhanced spectroscopy [3] or sensing [4].

In recent years, the development of nanoplasmonics has been carried out using mainly gold nanostructures due to their high plasmonic performance and excellent chemical stability [5]. However, due to its dielectric properties, silver has the highest plasmonic activity within all noble metals when used to build nanostructures and is also more economically attractive [6]. The main obstacles faced by silver-based nanostructures when dealing with different plasmonic applications are related to the silver's low chemical stability and poor wettability (i.e., the deposited atoms have greater tendency to bind to each other rather than to the substrate atoms, favoring 3-dimensional islands growth and as consequence increasing rough surface) on dielectric substrates which tend to diminish the substrates performance and limit their applications [6–8]. To overcome these challenges, 2 main approaches have been explored to generate chemically stable and flat silver plasmonic films on dielectric substrates: (1) the addition of a protective surface layer (i.e., self-assembled monolayers [SAMs] [9], oxides and other metals [10]) and (2) the use of a nucleation layer (i.e., germanium [11], chromium + gold [10] and polymers [12]). The addition of a protective thin gold film (Ag/Au bilayer system) can increase the chemical stability. However, under certain oxidative environments such as saline solutions, the chemical stability and reproducibility for long-term use can be also affected. This can be partly related to the low wettability (which implies high roughness) of silver on dielectric substrates and the mismatch adhesion of silver with different materials [8, 10]. To overcome these difficulties, the use of nucleation layers has been also proposed, but most of the materials employed (i.e., germanium, titanium and chromium) increase ohmic plasmon losses, thereby diminishing the final performance [13, 14]. Recently, another approach using a thin gold nucleation layer between a chromium adhesion layer and the upper silver layer has been demonstrated to successfully generate flat silver plasmonic films with improved chemical stability on glass substrates creating a 4 layer system (Cr/Au/Ag/Au) [9, 10].

The use of polymer-based substrates can increase the potential applications of plasmonic nanostructures, as the flexibility these materials can confer expand their use, for example, enabling the direct integration of sensors in the human body, or for solar energy harvesting, among others [15, 16]. As recently reported [17], the fabrication of plasmonic gold sensors using commercial Blu-ray discs as a flexible nanostructured polycarbonate polymer substrate (substrate with concentric periodic nanoslits of 320 nm period, 160 nm width and ≈20 nm depth) is a simple and highly reproducible process. Efficient deposition of thin gold films can be accomplished without the need of an adhesion layer (i.e., germanium, chromium, titanium) that can negatively affect the plasmonic performance in different ways [13, 14].

Based on these considerations, we have fabricated plasmonic sensor devices that combine trilayer (Au/Ag/Au) films and the precise nanostructured arrays of the commercial polycarbonate Blu-ray discs (see Appendix S1, Supporting Information, for details). The nanostructures were characterized and assessed from a biosensing perspective. A total metallic film thickness of 100 nm was selected for the fabrication of the devices as previous studies demonstrated that optimal plasmonic performance occurred at thicknesses between 50 and 120 nm. At metallic layers below 50 nm, the plasmonic reflectance spectra lose sharpness due to the strong interaction of plasmonic waves with the underlying substrate, whereas for metallic layers above 100 nm, no significant change in the plasmonic reflectance spectra is observed [17, 18]. For the adhesion/nucleation layer, different Au layer thicknesses were evaluated (from 0 to 5 nm), which seems to be sufficient to improve the adhesion of the silver layer while minimizing alterations to the plasmonic propagation due to a strong optical absorption of the adhesion/nucleation layer [19]. On the other hand, different thicknesses of top Au layer (5 -25 nm) were evaluated to improve the chemical stability of the substrate under high oxidative media (ultraviolet [UV]/O3 oxidation and a high salt content media) commonly used in biosensing assays. For comparison, we also fabricated Ag/Au bilayer systems without the Au nucleation layer.

The bare and the multilayered nanostructured surfaces were first characterized by atomic force microscopy (AFM). Figure 1 shows the surface roughness and the profile. Surface roughness values obtained in root-mean-square (RMS) were 0.21, 0.36, 1.29 and 0.65 nm for bare Blu-ray, Au, Ag/Au and Au/Au/Au surfaces, respectively. For the Au/Ag/ Au surface, a 2 nm Au nucleation layer increases the adhesion of Ag to the substrate, reducing the stratification and therefore decreasing the surface roughness compared to the Ag/Au surface (0.44 vs 1.08 nm, by subtracting the surface roughness of the bare nanostructured substrate) [9]. The roughness value for the Au/Ag/Au nanostructured polymeric substrate (0.44 nm) was lower than the one previously reported for Cr/Au/Ag/Au [9] and Ge/Ag [8] multilayer films, being all of them fabricated on flat glass substrates. These results emphasize that also for polymeric substrates, ultrasmooth plasmonic Ag-based nanostructures can be fabricated without using adhesion/nucleation materials, which significantly increase ohmic plasmon losses (i.e., germanium, chromium and titanium) [17].

The trilayered Au/Ag/Au nanostructured polymer substrate was also optically characterized with reflectance measurements, collecting spectra at different angles of incident light (30°-70°) with a transverse mode (TM)-polarized broadband light in air (η = 1.00) and water (η = 1.33) (see Figure S1). The observed shift in the resonance peak
position ($\lambda_{SPP}$) to higher wavelengths and the narrowing of resonant linewidths with high incident angles is correlated with the generation of Fano resonances [17]. By increasing the angle of the incident light, the optical energy scattered from one nanostructure can be collected by neighboring nanostructures decreasing radiative loss and increasing the plasmon lifetime, as previously reported [17] for Au nanoslits. The sensors integrated with a single channel flow cell (see Figure S2 for the sensing set-up) was used to evaluate the optical behavior of the multilayer sensors. The optical effect of different adhesion/nucleation Au layer thicknesses (0, 2 and 5 nm) was evaluated by observing the shift in the plasmonic resonance peak position ($\lambda_{SPP}$) when injecting different refractive index (RI) solutions (ie, glycerol solutions between 4.2 and 68 mM). As shown in the Figure S3, for the Au/Ag/Au system, an adhesion/nucleation Au thickness layer close to 2 nm seems not to affect the plasmonic propagation due to a strong optical absorption compared to a thicker Au layer (ie, 5 nm) and other adhesion/nucleation materials (ie, Ge, Cr and Ti) [8, 9, 20].

The optical characterization results were contrasted with those calculated from Finite-difference time-domain (FDTD) simulations (see Appendix S1 for details). As can be observed in Figure 2A, there is a good agreement between the calculated and the experimental reflectance spectra, with a narrower resonant linewidth for the Au/Ag/Au substrate compared to the Au substrate. As previously discussed, the dielectric properties of Ag provide nanostructures with higher plasmon field enhancements and narrower full width at half maximum (FWHM) spectra compared of those with only a Au monolayer. To evaluate the theoretical plasmon field enhancement in Au/Ag/Au and Au substrates, we analyzed the electric field distribution calculated from FDTD simulations. Figure 2C, D illustrates the plasmon enhancement in the electric field distributions of the Au/Ag/Au and the Au substrates, respectively. The higher plasmonic activity owing to the addition of a silver layer in Au/Ag/Au substrates is noticeable with an increase in the intensity of the optical fields compared to Au substrates [6, 9].

The effect of the top Au layer thickness in the chemical stability of the trilayered nanostructured plasmonic sensor was evaluated under high oxidative conditions (UV/O3 oxidation and a high salt content media) commonly used in biosensing assays and it was compared with bilayer sensors as
reference (no Au nucleation/adhesion layer). Multilayer sensors with 3 different top Au layer thicknesses (5, 15 and 25 nm) were placed in a UV/O3 generator for 10 minutes. A top Au layer thickness $\geq$ 15 nm provides a significant protection against oxidation. Moreover, the lack of the 2 nm nucleation layer (ie, bilayer sensors) results in a significant degradation even with a top layer of 15 nm, which makes clear the necessity of including this layer in the design (see Figure S4). The stability under aqueous oxidative media was evaluated by integrating the sensors with the flow cell and performing real-time tracking of the resonance peak position ($\Delta \lambda_{SPP}$) (see Figure S5). The stability under aqueous oxidative media was evaluated by integrating the sensors with the flow cell and performing real-time tracking of the resonance peak position ($\Delta \lambda_{SPP}$) (see Figure S5). A high salt content solution (PBS 100 mM with 1.4 M NaCl) was continuously flowed (see Appendix S1 for details). A steady baseline ($\Delta \lambda_{SPP}$) is observed for the trilayer Au/Ag/Au system as in the case of single Au layer substrates, which confirms its stability over time. Under the same experimental conditions, the bilayer system (Ag/Au) suffers a pronounced change in the $\Delta \lambda_{SPP}$. 

As expected, the improvement in adhesion and as consequence, the decrease of roughness of silver using the thin Au adhesion/nucleation layer, is correlated with the improved chemical stability of the Au/Ag/Au substrate compared to Ag/Au counterpart [9].

The performance of the trilayer plasmonic device for sensing applications was also evaluated with different glycerol solutions. Main parameters were extracted after injection of the different solutions: bulk sensitivity, FWHM and the resultant figure of merit (FOM) at different angles of incident light. The measurements were done also in real time, keeping as running buffer a constant flow of H$_2$O (30 $\mu$L/min). The results were compared to those previously obtained for the Au plasmonic sensor [17]. Figure 2B shows a comparison of the sensing performance parameters for both plasmonic devices. The Au/Ag/Au device resulted in better overall performance compared to Au-layered substrates at all the incident angle tested, being 70$^\circ$ the best one as previously reported [17]. Although the enhancement in sensitivity was around 12% (bulk sensitivity of 476 vs 425 nm-RIU$^{-1}$ in Au), the narrowing of the peak (with a FWHM reduced approximately 57%, from 12 to 7 nm) led to an enhancement of the FOM of a factor of 2 (from 34.9 to 69.2 RIU$^{-1}$ for the trilayered device).

This improvement was evaluated also by a biosensing assay on the trilayered Au/Ag/Au nanostructured sensor. A direct assay based on the attachment of specific antibodies and the detection of the corresponding target protein was
considered (see Appendix S1 for experimental details). The selected protein, C-reactive protein (CRP) is a well-known and valuable biomarker related with inflammation and infection processes. Au/Ag/Au and Au nanostructured surfaces were modified by forming a SAM with carboxylic acid, which was activated and further reacted with the specific antibody for CRP. The immobilization of the antibody was monitored in real time, as can be seen in the Figure S6. The detection of CRP at different concentrations (from 25 to 1000 ng/mL) shows a good dose—response (see Figure 3A). We were able to achieve a Limit of Detection (LOD) of 2.6 ng/mL (20.8 pM) and a Limit of Quantification (LOQ) of 9.1 ng/mL (72.9 pM), respectively (see the calibration curves in the Figure S7) which represents an improvement in biosensing performance compared to the Au single layer sensor (LOD of 3.7 ng/mL and a LOQ of 12.9 ng/mL). The higher plasmonic activity using a silver layer improves the biosensing performance of the device [9, 10]. The viability of measuring biological samples like undiluted urine was also assessed with these sensors. In this case, in order to minimize nonspecific adsorptions from components present in the complex media, an additional blocking step was included in the biofunctionalization protocol. A solution of poly(L-lysine) poly(ethylene glycol) (PLL-PEG) (0.5 mg/mL) was added to the antibody-immobilized sensor, as this reagent has previously demonstrated its effectiveness as antifouling agent [21]. Similar shift was observed for the same concentration of CRP in buffer before and after blocking (see Figure 3B, black and red lines), which confirms that this step does not affect the ability of the antibody to bind its target. The injection of pure undiluted urine and undiluted urine including a non-specific protein (ie, Bovine serum albumin (BSA)) resulted in no background ($\Delta\lambda_{SPP} = 0$) (see Figure 3B, green and magenta lines), which confirms the lack of nonspecific binding onto the biofunctionalized surface. Finally, the same CRP concentration in pure urine also resulted in the same signal obtained in buffer (same $\Delta\lambda_{SPP}$) (blue line in

**FIGURE 3**  Biosensing experiments for the Au/Ag/Au trilayer sensors. (A) Sensorgrams showing the detection of the target CRP protein at different concentrations (from 25 to 1000 ng/mL) in PBS buffer; (B) Blocking step and urine effect. Sensorgrams showing the detection of CRP (200 ng/mL) in PBS without PLL-PEG blocking (black line) and after PLL-PEG blocking (red line); nonspecific binding of undiluted urine (green line); nonspecific binding of control protein (BSA, 500 μg/mL) in urine (magenta line); detection of CRP (200 ng/mL) in undiluted urine (blue line); (C) Sensorgrams showing the detection of the target CRP protein at different concentrations (from 25 to 1000 ng/mL) in undiluted urine. (D) CRP calibration curves in PBS and undiluted urine. The error bars reflect the SD from 2 measurements.
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Materials and methods

Figure S1. Variation of reflectance spectra in air (η = 1.00) and water (η = 1.33) of trilayer (2 nm Au/83 nm Ag/15 nm Au) nanostructured plasmonic sensor varying oblique light incidence angle.
Figure S2. Experimental set-up including the optical detection scheme and the microfluidic system. The detection scheme consists of reflectance measurements using a compact broadband light source under TM-polarization; the reflected light was collected and fiber-coupled to a compact charge-coupled device (CCD) spectrometer. The microfluidic delivery system consists of a syringe pump, with adjustable pumping speed, and a manually operated injection valve. Inset: Photography of the integrated nanoplasmonic sensor with the microfluidics flow cell

Figure S3. Real-time changes in the resonance peak position ($\lambda_{SPP}$) for different solutions of glycerol (angle of light incidence = 70°) for 3 multilayer structures with different adhesion/nucleation Au layer thicknesses: (black) no adhesion layer, (red) 2 nm Au and (blue) 5 nm Au

Figure S4. UV/O3 effect on multilayer sensors with different top Au layer thicknesses (5, 15 and 25 nm). (a) Trilayer samples with a 2 nm adhesion/nucleation Au before UV/O3 treatment. (b) Trilayer samples with a 2 nm adhesion/nucleation Au layer thickness after UV/O3 treatment (10 min). (c) Reference bilayer samples (without adhesion/nucleation Au layer) after UV/O3 treatment (10 min).

Figure S5. Real-time changes in the resonance peak position ($\lambda_{SPP}$) in a continuous flow of highly concentrated PBS (100 mM with 1.4 M NaCl) for the 3 different structures: Au monolayer, Ag/Au bilayer and Au/Ag/Au trilayer

Figure S6. Real-time sensorgrams showing the 3 steps involved in the covalent immobilization of the specific antibody anti-CRP over Au monolayer (black line) and Au/Ag/Au trilayer substrates (red line): (1) activation of carboxylic SAM layer with EDC/NHS, (2) attachment of the antibody and (3) blocking of unreacted active carboxylic groups with an ethanolamine solution

Figure S7. Calibration curves for CRP detection for the Au monolayer and Au/Ag/Au trilayer sensors. The error bars reflect the SD from 2 measurements

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