Influence of the nanoscale structure of gold thin films upon peroxidase-induced chemiluminescence

Guowei Lu, Bolin Cheng,[a] Hong Shen, Zhenghao Chen, and Guozhen Yang
Beijing National Laboratory for Condensed Matter Physics, Institute of Physics, Chinese Academy of Sciences, Beijing 100080, China

Christophe A. Marquette and Loic Jean Blum
Laboratoire de Génie Enzymatique et Biomoléculaire, Université Claude Bernard Lyon I, 69622 Villeurbanne Cedex, France

Olivier Tillement, Stéphane Roux, and Gilles Ledoux
Laboratoire de Physico-Chimie des Matériaux Luminescents, Université Claude Bernard Lyon I, 69622 Villeurbanne Cedex, France

Armel Descamps and Pascal Perriat
Groupe d'Etude de Métallurgie Physique et de Physique des Matériaux, INSA de Lyon, 69621 Villeurbanne Cedex, France

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Gold thin films with different nanoscaled roughness were elaborated by a pulsed-laser deposition technique in order to evaluate their ability to form biochip substrates. The crystal structure, microstructure, and optical absorption were investigated by x-ray diffraction, atomic force microscopy, and ultraviolet-visible absorption spectrum. Controlling the temperature of the substrate during the deposition process allows us to obtain samples with different roughness and grain sizes. The temperature can then be adjusted to elaborate thin films, which have either the optical behavior of bulk metal or that of individual clusters. This optical behavior strongly influences the chemiluminescence at 425 nm of luminol brought to the vicinity of peroxidase supported by biomolecules physi- or chemisorbed on the films. In particular, the signal intensity increases of almost one order of magnitude when the film presents a significant surface resonance plasmon.
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Biosensors and biochips of highly sensitivity and selectivity have been greatly improved by the development of gold nanoparticles and thin films. Gold colloids as direct or indirect probe are applied to achieve efficient electrical, colorimetric, or surface enhanced Raman scattering biological detection.[1] Gold thin films are also widely used as chip substrates or electrodes to immobilize organic molecules via the helpful Au—S covalent bond.[2] For example, the surface plasmon resonance (SPR) of gold thin films was used to detect the dielectric constant changes induced by the molecular adsorption at the surface of the films.[3] However, the use of gold films to design devices liable to detect the specific adsorption of molecules by the luminescence of labeling dyes has been rarely reported. This is probably due to the fact that dye fluorescence is quenched in contact with metallic beads.[4,5] This quenching is due to the re-absorption of the dye emission by the gold surface plasmon according to a resonant energy transfer process.[6] Compared with this background, the present letter shows a completely different result: in some optimized conditions the roughness can enhance instead of quench the luminescence of luminol brought to the vicinity of gold films. This result is consistent with a recent paper that reports an enhancement of luminol electrochemiluminescence when bulk Au electrodes are replaced by gold nanoparticles self-assembled electrodes.[7] Here, we elaborated a series of gold thin films with different nanoroughness by using a pulsed-laser deposition (PLD) technique. We evidenced a clear effect of the nanostructure of the film upon the chemiluminescence (CL) of luminol, thus opening a route for developing a new kind of biochip.

All the gold films were deposited by a pulsed-laser deposition system in vacuum (~10 Pa) onto fused quartz substrates with a severe control of key parameters, such as the energy of the laser, the distance between the substrate, and the target and the temperature of the substrate, which are known to strongly influence films nanostructure.[8] The morphology of films having the same nominal thickness of around 25 nm was tuned by varying the substrate temperature from 30 to 700 °C. Atomic force microscopy images of the gold thin films [Fig. 1(a)] revealed that flat and smooth films are elaborated when the substrate is maintained at room temperature, whereas clustered surfaces are obtained when the substrate is heated at temperatures higher than 200 °C [Fig. 1(b)]. In the case of a substrate heating, the roughness and particles size of the clustered films increases with increasing the substrate temperature (Table I). Roughness is defined as the standard deviation of the height value within a box cursor with a size of 250×250 nm^2 and a resolution of 256×256. Particle size is estimated from an average on more than 30 particles. As in vacuum thermal evaporation technique,[9,10] a similar influence of the substrate temperature upon roughness and particles size is observed for both methods. Here, PLD allows us to obtain a large variety of film morphologies, especially either cluster-like or bulk-like films.

The gold films were also characterized by optical absorption measurements made at room temperature from 330 to 800 nm using a SpectraPro-500i spectrophotometer (Acton Research Corporation). Typical absorption spectra are

[a]Electronic mail: blcheng@aphy.iphy.ac.cn

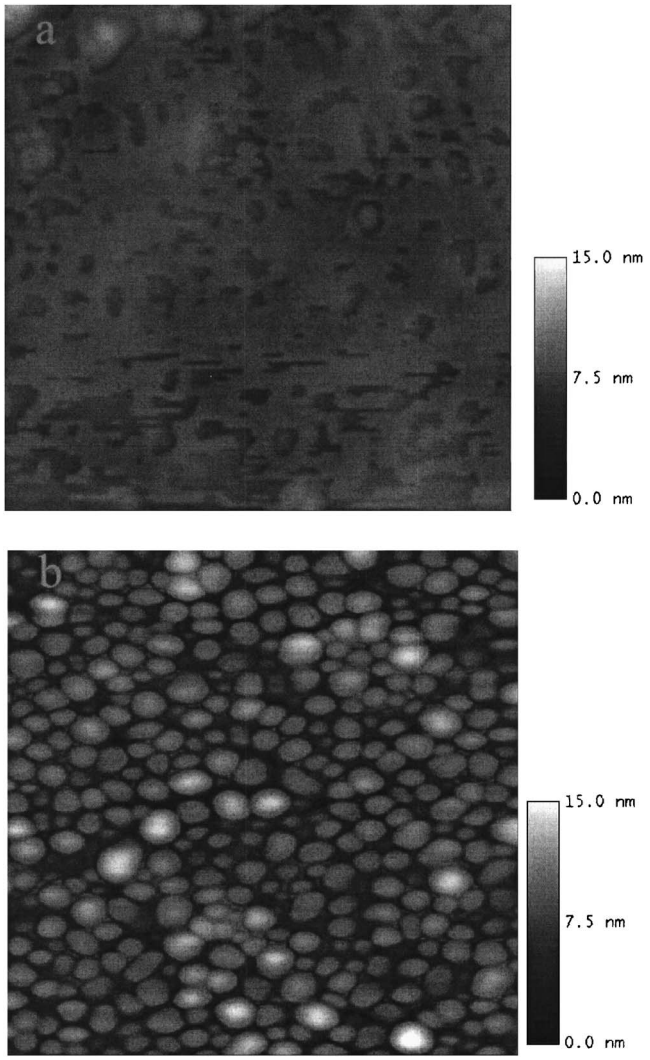

FIG. 1. AFM images of gold films, prepared at (a) room temperature, and at (b) 400 °C (area of 500 nm×500 nm).

shown in Fig. 2. The position and the half-width at half-maximum (HWHM) of SPR are indicated in Table I. Again, the results allow to classify the films in two categories: in absence of heating, the films exhibit the mean features of the absorption of gold bulk,[11] whereas for heating at temperatures higher than 200 °C the films present a pronounced SPR extinction peak characteristic of the presence of clusters.[12] This peak is located at about 600 nm and, as expected from the roughness increase observed by atomic force microscopy (AFM),[9,10] has a blueshift with increasing the temperature of the substrate.

TABLE I. The peak of SPR wavelength, HWHM, roughness, and particle size of gold thin films.

Temperature (°C)	SPR peak (nm)	HWHM (nm)	Roughness (nm)	Particle size (nm)
30	No peak	No peak	0.3	Non
200	670	110	1.3	~15
300	635	75	1.5	~20
400	625	72	1.5	~25
500	625	70	1.6	~30
600	620	72	1.9	~35
700	610	62	2.0	~50

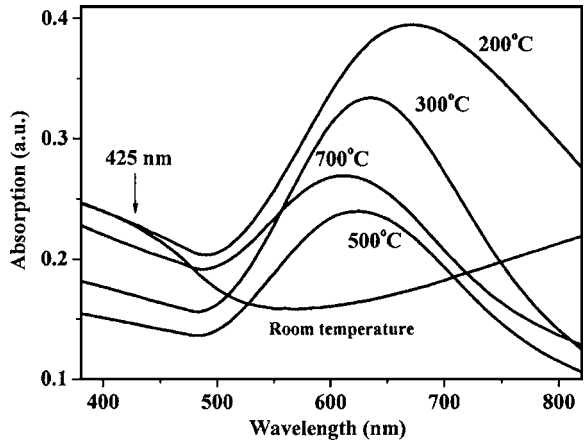

FIG. 2. Absorption spectrum of gold films for different substrate temperatures: 30, 200, 300, 500, and 700 °C, respectively.

In order to evaluate the ability of the gold substrates to serve as biochips, physisorption and chemisorption of peroxidase labeled biological molecules acting as catalyst for CL were performed on the films.[13] To realize physisorption, four 1.2 nl drops (2 μg/ml) of peroxidase labeled streptavidin with globular size 6–10 nm (Sigma, France) were deposited through an automatic piezoelectric spotter (BCA1, Perkin Elmer) at the surface of the film, generating a 2×2 spot matrix according to a process described in Fig. 3(a). The labeled streptavidin was stand to physisorb for 2 h at room temperature before a washing for 20 min in 30 mM Veronal buffer *p*H 8.5 containing 0.2 M NaCl (VBS). To realize chemisorption, peroxidase labeled streptavidin was fixed after previous immobilization on the films of a trithiolated peptide (12 amino acid from Epytope, France, the linear size <2 nm) modified with a biotin molecule at its N-term end. This process is described in Fig. 3(b). In a first step, the peptide was spotted as 1.2 nl drops (1 mg/ml), incubated during 2 h, and washed to remove all unbound molecules. In a second step, the Au thin films were immersed for 20 min in VBS containing in addition 1% bovin serum albumin and 0.1% polyoxyethylenesorbitan monolaurate (tween) and incubated 30 min with peroxidase labeled streptavidin (1 μg/ml). CL measurements were taken under a −30 °C cooled CCD camera (Intelligent Dark Box II, Fuji Film). Briefly, the treated thin films were dipped into a VBS solution containing luminol and additives agents favoring its CL

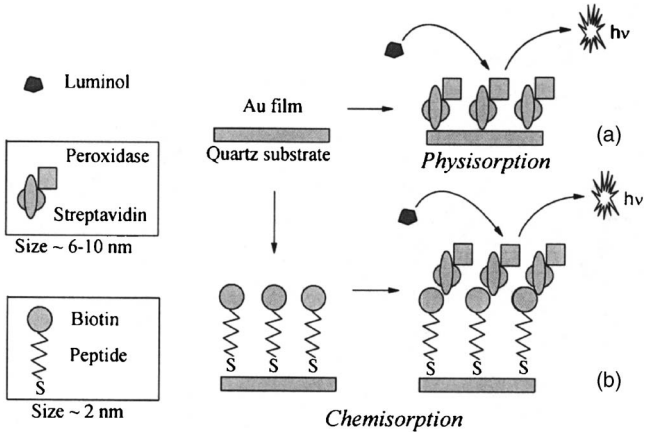

FIG. 3. Schematic representation of the process involved in luminol chemiluminescence induced by peroxidase labeled molecules physisorbed (a) and chemisorbed (b).

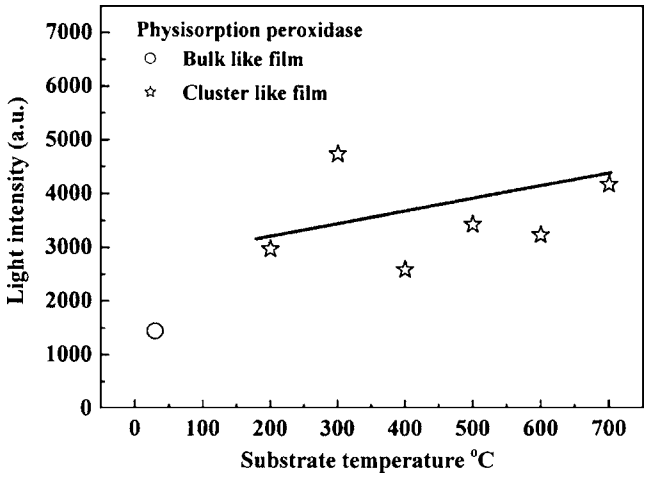

FIG. 4. Chemiluminescence intensity induced by peroxidase labeled molecules physisorbed on substrates made at different temperatures: 30, 200, 300, 400, 500, 600, and 700 °C.

(220 μM luminol, 500 μM H_2O_2, and 200 μM p-iodophenol). The light emitted by the luminol brought to the peroxidase vicinity was integrated for 10 s. The pictures obtained were quantified and the results given in arbitrary units (a.u.). Each value is an average of four measurements.

Whatever the nature of the peroxidase adsorption, of a physical or a chemical type, the evolution of the CL intensity always presents two distinct features. First, the luminescence is considerably stronger for the films, which present the optical properties characteristic of clustered samples. This is verified in Fig. 4, which displays the CL intensity of luminol brought at the vicinity of physisorbed peroxidase on different films. It is even clearer in Fig. 5, which shows that, when peroxidase is chemisorbed, the CL enhancement is of more one order of magnitude. Precisely, the intensity of the luminescence emitted by the luminol is around 1700 for the bulk-like film prepared at 30 °C, whereas the intensity is between 17 000 and 26 000 for the films prepared above 200 °C for which a clear surface plasmon is observed. The second feature concerns only the samples that present a cluster-like behavior. It consists of a slight but clear increase of the chemiluminescence with the substrate temperature. This slight increasing feature is observed regardless of the nature

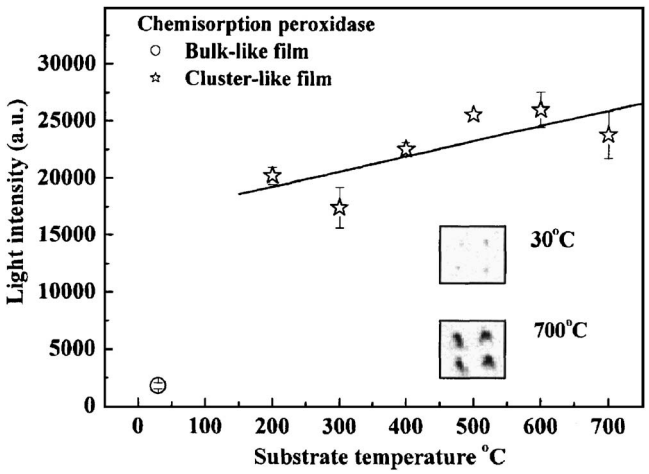

FIG. 5. Chemiluminescence intensity induced by peroxidase labeled molecules chemisorbed on substrates made at different temperatures: 30, 200, 300, 400, 500, 600, and 700 °C. Insets: chemiluminescence spots for substrates made at RT and 700 °C, respectively.

of the peroxidase adsorption, because of increase of the specific area of the films, which induces a correlative increase of the amount of peroxidase adsorbed.

In this letter, the luminol was chosen as the luminescent entity since it emits at 425 nm, a wavelength which is not re-absorbed by the gold surface plasmon situated above 600 nm. The absence of CL quenching could be also related to the distance between luminol and gold (of the same order of magnitude that between peroxidase and gold: ~10 nm), which is here larger than the Forster distance (a few nanometers) required to achieve an efficient resonance energy transfer.[14] Finally, the CL enhancement observed is certainly due to the catalysis by gold clusters of reactions involving peroxidase or one of the complexes or radicals formed from it.[15] The need of peroxidase remains essential in the whole CL process since, whatever the enhancement, CL is only observed at peroxidase vicinity (inset of Fig. 5). This explanation is consistent with the fact that gold clusters act as strong catalyst in solution.[16] They are indeed known to contribute both to the formation of active oxygen-containing reactant intermediates[17] and to the stabilization of these intermediate radicals via partial electron exchange interactions.[18]

In summary, we elaborated gold thin films with nanometer-scale roughness by controlling the parameters of the PLD technique. When the films support a catalyzer of luminol chemiluminescence (the peroxidase), a corrugation of the films generates an emission enhancement of the luminol brought to proximity. This observation contrasts with previous results that all evidenced dyes quenching in presence of gold nanoparticles or corrugated surfaces as an effect of the re-absorption of the dye emission by the surface plasmon. The complete understanding of this phenomenon should open a promising route for developing new devices for biological detection.

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