How the morphology of biochips roughness increases surface-enhanced chemiluminescence

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Abstract

Biochips of Au particle arrays were fabricated using nanosphere lithography to investigate the influence of gold roughness upon chemiluminescence induced by peroxidase adsorbed at surface. The corrugation of gold induces a strong enhancement of the chemiluminescence of luminol brought at its vicinity. This letter shows that this enhancement is increased by an order of magnitude when the corrugation is regular and possesses an average curvature radius of around 15 nm (for ordered particles arrays compared to films with random roughness).
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There has been a continued interest in the specific interactions between metal surfaces and nearby molecules or particles in the last decades [1,2]. Such investigations not only make new insights into the basic aspects of field-matter interaction [2] but also give opportunities to develop ultrasensitive analytical techniques for bioanalytical applications [3,4]. The most celebrated effect associated with metal/molecule interactions is the surface enhanced Raman scattering, which has great potential for single-molecule-detection [5]. Additionally, it has been established that rough metal films or metal particles have dramatic effects on the fluorescence behavior of fluorophores and nanocrystals nearby, including modified fluorescence intensity, decreased lifetimes, increased photostability and directional emission. Such metal-enhanced fluorescence, which has attracted more attention in recent years, promises to provide the next generation of high sensitivity fluorescence

assays for low copy number detection of biochemical species [6–8]. It is worth to mention that chemiluminescence (CL) and electrochemiluminescence are very effective detection methods among the luminescence-based systems for bioanalysis and immunoassay because the emission occurs without autofluorescence from the samples [9,10]. In this context, metallic surfaces and particles can be used to increase the sensitivity of the chemiluminescence-based assays [11,12]. In a previous work, we have studied the effect of a nanometer corrugation on the CL of luminol brought at the vicinity of a gold film [13]. Comparatively to flat films, gold films with random roughness allow to enhance the luminol CL by a factor of ten. The enhancement observed was attributed to the catalysis by gold clusters of reactions involving peroxidase. In order to further optimize the CL enhancement, investigations should be carried out on the influence of the surface morphology. In this letter, we demonstrate that a well-controlled roughness allows increasing the CL enhancement by an additional order of magnitude. Such a controlled roughness was achieved by elaborating well-ordered arrays consisting

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of homogenous Au nanoparticles. Two kinds of isolated particles differing from their diameter were studied leading to the conclusion that the CL enhancement was stronger for particles with a radius of around 15 nm.

The ordered gold nanoparticle arrays were fabricated using nanosphere lithography [14], an inexpensive, simple and high throughout nanofabrication technique. Firstly, a mask consisting of a single or a double-layer of polystyrene (nano)spheres was deposited on glass substrates by a self-assembly process. Briefly, 30 µL of diluted nanosphere solution (1 wt% solid, diameter of 200 nm) was dropped onto a cleaned glass substrate that was inclined between 4° and 6° in a chamber with saturated humidity. By controlling the temperature between 35 °C and 45 °C, we successfully formed homogenous and dense single or double-layers of nanospheres on areas larger than 1 cm². After drying, gold films with different thickness were deposited on the substrates at room temperature (RT) using a pulsed laser deposition (PLD) technique. After complete removal of the nanosphere masks by sonication in chloroform, gold nanoparticle arrays with 2D periodic structures were obtained. For comparison, Au flat and corrugated films were also deposited on glass substrates in the same PLD system at 25 °C and 600 °C, respectively. The AFM images of Au films prepared at 25 °C and 600 °C are shown in Fig. 1a and b which display the smooth and randomly islandized surfaces, respectively. The average particle size of the randomly corrugated film is estimated to be about 35 nm. Fig. 1c gives the image of particle arrays fabricated using a single-layer nanosphere mask (SL Au). They show a well-ordered hexagonal array belonging to the p6 mm

plane group. Each cell contains two triangular-shaped gold particles with a size of 50 nm (a diameter of 200 nm was chosen for the polystyrene spheres to ensure that particles size is of the same order of magnitude than for corrugated film). Fig. 1d exhibits the arrays fabricated using a double layer nanosphere mask (DL Au). The hexagonal array belongs to the same p6 mm plane group but each cell contains only one particle with a smaller size compared to SL arrays. The shape of the particles should be consistent with that of the interstices let by the polystyrene nanospheres i.e., triangular and hexagonal for SL Au and DL Au arrays, respectively. In fact, the particle shape was smoothed slightly during the fabrication process as evidenced in the AFM images so that, in the case of DL Au arrays, the particles appear almost circular.

The extinction properties of all the films were investigated using a SpectraPro-500i spectrophotometer (Acton Research Corporation). All spectra were collected in standard transmission geometry with unpolarized light and a probe beam diameter of 1 mm. When visible light penetrates particles or island films [15], a localized surface plasmon resonance (LSPR) corresponding to a coherent oscillation of the spatially confined electrons is induced [16]. Surface plasmon polariton (SPP) are also excited if the metal film presents some corrugation, the excitation being maximal when the roughness periodicity matches the wavelength of the incident light [17]. For a randomly corrugated film, all periodicities are present ; then SPP could be theoretically excited by any conventional illumination. However, the ill-defined SPP excitation condition results in a very low efficiency of the non-resonant
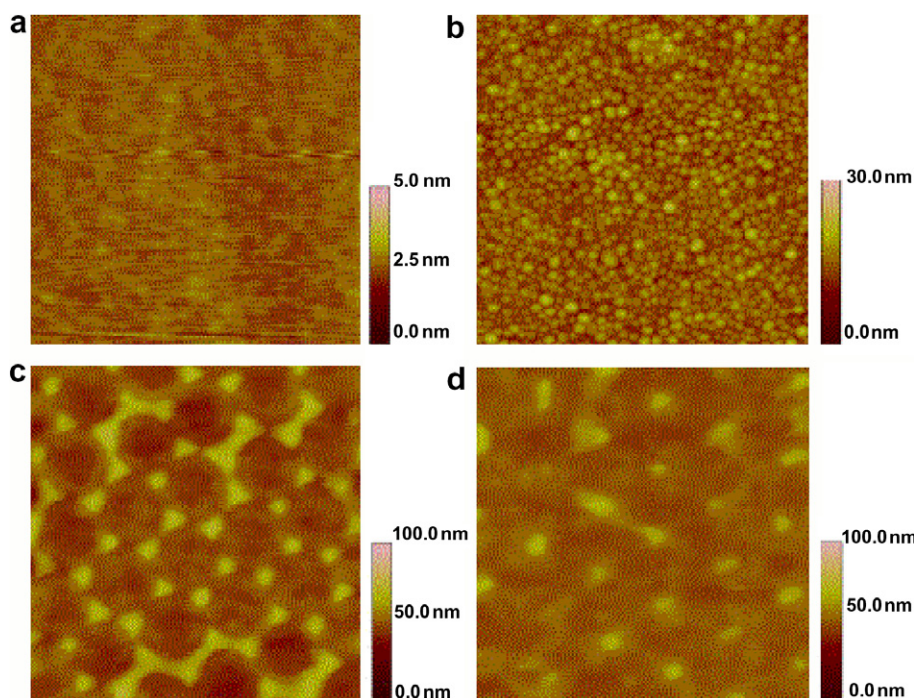

Fig. 1. AFM images of the different films elaborated by PLD. Films prepared without template at (a) 25 °C and (b) 600 °C. Films fabricated by nanosphere lithography using a single (c) and a double (d) layer sphere mask.

light-to-SPP coupling [16] so that, for the randomly corrugated films studied in this letter, only LSPR should be considered. For particle arrays, the metal dots are deposited on a non conducting quartz substrate. Thus SPP can not propagate [17] and LSPR is again the only plasmon to consider. As shown in Fig. 2a, two resonance peaks are observed for SL Au arrays in the range [400–1000 nm] whereas only one (broad) peak is observed for DL Au arrays. This behavior can be explained by the difference in shape between the individual particles obtained in both cases. For SL arrays, the particles are highly anisotropic (see Table 1) so that the different geometrical depolarization factors found in- and out-of-plane lead to two separate resonances. It is worthy to note that when the anisotropy decreases (from SL Au 17 to SL Au 33), the two resonance peaks tend to merge. For DL arrays, the shape of the particles is smoother and only slightly anisotropic so that only a broad peak is observed. Also Fig. 2b shows that the extinction spectrum of the Au flat film exhibits the features of bulk gold while the corrugated film exhibits a pronounced peak at about 615 nm. Compared to free clusters, the resonance is redshifted due to electromagnetic coupling effects between

neighboring islands [18]. All the characteristics of the different films are indicated in Table 1.

In order to use the metal films made so as chip substrates for luminol-H_2O_2 induced CL, peroxidase was fixed on the films according to a process described in Fig. 3. In our previous work, the CL enhancement was found to strongly depend on the adsorption type of peroxidase (physical or chemical) [11]. Since this difference is now thought to originate from a variation in the distance between peroxidase and metal, we decided in this study to control precisely this distance by using two tri-thiolated polypeptides P5, P23 with different chain lengths (1.5 nm and 8 nm, respectively). These polypeptides differ from their number of elemental peptides (5 for P5 and 23 for P23) but both have a sequence of tri-thiolated cysteine at C extremity and are conjugated to biotin at N extremity. These two polypeptides (P5, P23) permit to separate the peroxidase from the Au surface of about 4.5 nm and 11 nm, respectively (when taking into account the size of the peroxidase which is of about 6 nm). Firstly, the tri-thiolated polypeptides were spotted as 1.2 nl drops (1 mg/ml) on each of the films through an automatic piezoelectric spotter (BCA1, Perkin Elmer). The peptides were incubated during 2 h and then washed to remove all unbound molecules. In a second step, the treated substrates were immersed for 20 min in veronal buffer solution (VBS) containing additional 1% bovin serum albumin (BSA) and 0.1% polyoxyethylenesorbitan monolaurate (tween) and finally incubated with peroxidase labeled streptavidin (1 µg/ml) for 30 min.

Chemiluminescence (CL) measurements were taken with a −30 °C cooled CCD camera (Intelligent Dark Box II, Fuji Film). After immobilization of labeled streptavidin, the substrates were dipped into a VBS solution containing luminol and additives agents favoring its CL (220 µM luminol, 500 µM H_2O_2 and 200 µM *p*-iodophenol). The light emitted by the luminol brought at the peroxidase vicinity was integrated for 10 s. The pictures obtained were quantified and the results were given in arbitrary units (a.u.). Each value is an average of four measurements.

The CL intensity of luminol at the vicinity of peroxidase on the glass, flat and corrugated Au films and particle arrays are shown in Fig. 4. Much stronger luminol CL is observed with metal films presenting some corrugation (random or periodic). It is consistent with our previous work that already demonstrated that a rough metal surface could significantly enhance luminol CL. It is also in agreement with the general observation that, even if bulk gold is chemically inert, gold particles turns out to be surprisingly active for many reactions [19–21]. The present results show now that the CL intensity is also strongly dependent on the distance between peroxidase and metal. A strong quenching due to a nonradiative energy transfer between fluorophores and metal is expected for distances smaller than 5 nm [6–8]. That explains why the CL measured is low at small metal/peroxidase distance (P5) and more important at larger distance (P23) at which quenching is found to
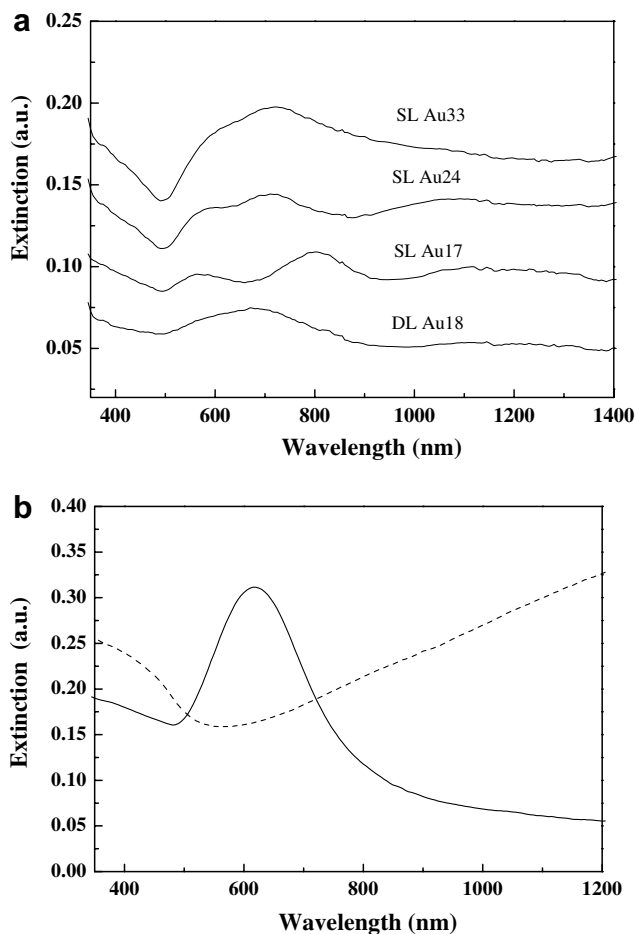

Fig. 2. Extinction spectra of nanoparticle arrays fabricated with monolayer (SL Au 17, 24 and 33) or double layer (DL 18) nanosphere masks (a) and of flat (dashed line) and rough (solid line) Au films (b).

Table 1
Characteristics of the films elaborated

Samples		In-plane particle size (nm)	Out-of-plane particle height (nm)	SPR peak (nm)	SPR intensity	CL intensity (a.u.)	Au surface ratio (%)
Nanoparticle arrays	SL Au17	50	17	565–802	0.08	17800	25.5
	SL Au24	50	24	83–715	0.09	20300	32.7
	SL Au33	50	33	620–722	0.10	19700	42
	DL Au18	30	18	667	0.05	8860	8.8
Flat Au film	Au RT	/	25	No peak	/	1800	100
Rough Au film	Au 600	35	25	620	0.23	20300	120

'Au surface ratio' denotes the gold area normalized to that of supporting glass.
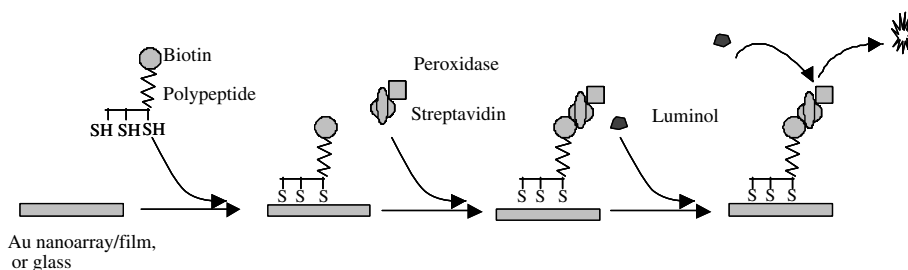

Fig. 3. Schematic representation of the process involved in luminol chemiluminescence induced by peroxidase labeled molecules.
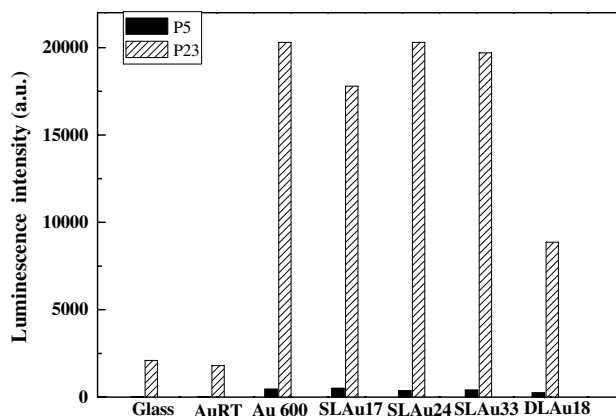

Fig. 4. Chemiluminescence intensity of luminol at the vicinity of peroxidase on the glass, flat Au film, corrugated Au film and nanoparticle arrays.

Fig. 5. Chemiluminescence intensity per unit area of Au on the flat and corrugated films and nanoparticle arrays.

be completely negligible [22]. Whatever the metal/peroxidase distance, the enhancement induced by corrugation is of one order of magnitude and even slightly stronger for the shorter distance: in arbitrary units indeed, CL increases from 20 for the flat film (AuRT) to 450 and 500 for the randomly (Au600) and periodically (SLAu17) corrugated films in the case of the shorter distance (P5) and from 1800 to 20 000 and 18 000 in the case of the larger one (P23).

To evidence an eventual effect of the nature of corrugation upon luminol CL, this latter must be normalized by the effective gold surface area which strongly differs between the randomly corrugated films (for which gold covers all the substrate surface) and the particles arrays
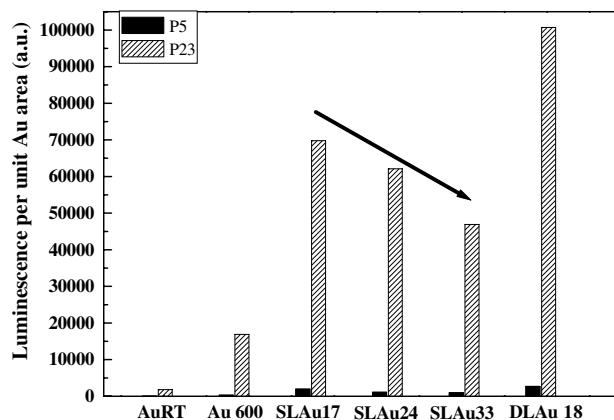
(for which gold only lies in small and isolated islands). Fig. 5 shows that the area-normalized luminescence is of almost one order of magnitude higher in the case of the particles arrays, which demonstrates that the roughness morphology of gold is a key parameter for enhancing CL. The mechanisms responsible for such an enhancement could be related either to a plasmonic enhancement or to a catalytic effect. The plasmon-assisted enhancement, promoted by a modification in photonic mode density [23], would yield to a strong increase in the low quantum efficiency of the CL agent. However, the authors having already observed such a CL enhancement [24] rather attributes it to a catalytic effect. According to them,

CL enhancement is mainly due to the interaction of Au clusters with the reactants or intermediates of the reaction of luminol-H_2O_2 system [25] involving peroxidase. In this process, the electrons at the vicinity of highly curved surface are the ones which cause some electron transfer from gold clusters to adsorbed H_2O_2 (a process known as particle-mediated transfer) [24] and permits to produce the key intermediate hydroxy and hydroperoxide radicals which leads to the enhancement of the CL [26]. A number as large as possible of these 'surface electrons' is then desirable to achieve the greatest catalytic efficiency. Since the intensity of the *Surface* Plasmon Resonance band is considered to be an indicator of the density of these *surface* electrons, [27] the results that we obtained should show a correlation between CL enhancement and SPR intensity [17] (these two independent phenomena being both favored in a same way by roughness). This is roughly what we obtain in the case of particle arrays since CL intensity (Table 1 and Fig. 4) varies then in a similar way than SPR intensity (Table 1 and Fig. 2a).

For particles, the size inducing the largest SPR is comprised between 20 nm and 40 nm [17]. Applied to corrugated films this indicates that the ideal roughness permitting to obtain a large plasmon resonance should be characterized by curvature radii half of this particle diameter: i.e., in the [10–20 nm] range. The randomly corrugated film which possesses a roughness of ≈2 nm is characterized by curvature radii of a few nanometers at particles junction. These curvatures are then too small to induce a large SPR intensity, which explains a (relatively) low CL enhancement. However for the particle arrays studied in this letter, the top of the dots is characterized by curvature radii that are approximately half of the particles in-plane diameter given in Table 1 (25 nm for SL arrays, 15 nm for DL arrays) and then lie in the ideal range inducing a maximal SPR intensity. That should explain why the CL enhancement is much more pronounced and, precisely, why it is greater for the DL array whose curvature radii correspond exactly to those leading to the maximum of plasmon resonance (15 nm). Very instructive also is that for the SL arrays (which all have a same in-plane diameter of 50 nm) the CL luminescence does not increase with particle height (Fig. 4) – and correlatively that the normalized CL decreases with increasing particle height (Fig. 5). This suggests that the upper part of the gold dots deposited on the substrate corresponds to the only region of gold allowing efficient catalysis of luminol CL which is consistent with the fact that only the top of the dots possesses a curvature radius suitable for inducing some significant

resonance. Indeed, the particles are triangularly shaped so that in the bottom of the particles, radii of curvature are certainly far smaller, in the 2–4 nm range according to Fig. 1c.

In summary, hexagonal Au particles arrays were fabricated and evaluated as biochips using peroxidase-induced CL of luminol. For different metal/peroxidase distances, they induce a CL enhancement of almost one order of magnitude greater than for randomly corrugated films. It emerges from the series of the samples studied that only electrons at vicinity of a surface with a curvature radius of around 15 nm are efficient for catalysis. This result gives way to further optimize the arrays structure in order to still increase their catalytic capacity.

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