Nanostructured gold surfaces as biosensors: surface-enhanced chemiluminescence and double detection by surface plasmon resonance and luminescence

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By
Meigui OU

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<table>
<thead>
<tr>
<th>Rapporteur</th>
<th>Supervisor</th>
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<td>C. LOUIS</td>
<td>P.PERRIAT</td>
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<tr>
<td>S. BEGIN</td>
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<td>J.L. BIEJON</td>
<td></td>
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<tr>
<td>O. TILLEMENT</td>
<td></td>
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<tr>
<td>S.ROUX</td>
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<tr>
<td>C.Marquette</td>
<td></td>
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<tr>
<td>M.B.VILLIERS</td>
<td></td>
</tr>
<tr>
<td>Director of research (CNRS)</td>
<td>Professor (INSA-Lyon)</td>
</tr>
<tr>
<td>Professor (ULP, Strasbourg)</td>
<td>Professor (UCBL)</td>
</tr>
<tr>
<td>Professor (UTT)</td>
<td>M.C. (UCBL)</td>
</tr>
<tr>
<td>C.R (CNRS)</td>
<td>C.R. (INSERM)</td>
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Laboratory
Matériaux : Ingénierie et Science, UMR CNRS 551
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**List of Abbreviation**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscope</td>
</tr>
<tr>
<td>APTES</td>
<td>Aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EBL</td>
<td>Electron Beam Lithography</td>
</tr>
<tr>
<td>EELS</td>
<td>Electron Energy-Loss Spectrometry</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>HAADF</td>
<td>High Angle Annular Dark Field</td>
</tr>
<tr>
<td>LSPR</td>
<td>Local Surface Plasmon Resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NSL</td>
<td>Nanosphere Lithography</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon Correlation Spectroscopy</td>
</tr>
<tr>
<td>PLD</td>
<td>Pulse Laser Deposition</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum Dots</td>
</tr>
<tr>
<td>RBITC</td>
<td>Rhodamine B Isothiocyanate</td>
</tr>
<tr>
<td>SECL</td>
<td>Surface-enhanced Chemiluminescence</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface-enhanced Raman Spectroscopy</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethyl orthosilicate</td>
</tr>
<tr>
<td>VBS</td>
<td>Veronal Buffer Solution</td>
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<tr>
<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
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Abstract

Nanostructured gold surfaces can be used as substrates of biosensor in biology due to their plasmonic and catalytic properties. This thesis is devoted to develop two biological detection systems based on nanostructured gold surfaces to detect the binding of streptavidin/avidin. In the first part, we have studied a multimodal detection system using local surface plasmon resonance of the gold substrate and the luminescence of labelling core-shell Gd$_2$O$_3$/SiO$_x$ nanoparticles, which profits from the plasmonic property of nanostructure gold. The synthesis of core Gd$_2$O$_3$ and core-shell Gd$_2$O$_3$/SiO$_x$ nanoparticles were optimized and the nanoparticles were characterized by Transmission Electron Microscopy and Electron Energy Loss Spectrometry.

In a second part, we have focused in a surface-enhanced chemiluminescence system based on chemiluminescence of luminol/hydrogen peroxide (H$_2$O$_2$) enhanced by gold nanostructures in the vicinity, which benefits from the catalytic property of nanostructure gold. In order to understand the mechanism of enhancement of chemiluminescence, several parameters influencing the chemiluminescence of luminol were investigated, such as the distance between substrate and peroxidase (catalyst of luminol), the nanostructure of gold substrate and pH value of solution. Ultimately, enhancing mechanism of luminal chemiluminescence was proved to be not related to Plasmon-assisted process but originates from catalytic properties of the metal induced by corrugation.

Keywords: surface-enhanced chemiluminescence, nanostructured gold, local surface plasmon resonance, core-shell Gd$_2$O$_3$/SiO$_x$ nanoparticles.
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Résumé

Depuis quelques décennies, le développement de biocapteurs devient un problème extrêmement important pour le diagnostic et la surveillance des maladies, la protéomique et la détection environnementale d'agents biologiques. Le premier biocapteur étudié était fondé sur la résonance des plasmons de surface (SPR) et pouvait détecter des anticorps en observant le changement de l'angle critique de réflexion lorsque ceux-ci se lient sélectivement à une couche d’or. Les capteurs SPR qui sont sensibles à toute modification de l’indice de réfraction ont ensuite été utilisés pour détecter la fixation d’une substance à analyser sur ou à proximité d’une surface métallique : l’adsorption de petites molécules ou de protéines sur des monocouches auto-assemblées, l’hybridation de l’ADN et les interactions entre l’ADN et diverses protéines. Différents groupes se sont également intéressés aux capteurs à base de résonance des plasmons de surface localisés (LSPR) qui sont sensibles à un changement localisé de l’indice de réfraction après adsorption de molécules biologiques. Les capteurs SPR et LSPR se distinguent par leur sensibilité à l’indice de réfraction : $2 \times 10^6 \text{ nm / RIU}$ (Unité d’indice de réfraction) et $2 \times 10^2 \text{ nm / RIU}$ respectivement et leur longueur d’évanescence du champ électrique : $l_d = 200-300 \text{ nm}$ et $l_d = 5-30 \text{ nm}$.

Une autre technique utilisée pour la bio-détection est l’exaltation de la diffusion Raman par une surface (SERS). Elle est caractérisée en ce que des molécules spécifiques sur ou à proximité d’une surface métallique présentant une rugosité à l’échelle nanométrique induisent une forte augmentation de l’intensité de lumière diffusée inélastiquement. Depuis les années 1980, ce phénomène a abouti à un nombre croissant d’applications biologiques comme l’imagerie moléculaire in vivo du cancer. Après la découverte de cet effet, d’autres phénomènes d’exaltation (SE) ont été mis en évidence au cours des dernières décennies comme la fluorescence exaltée de surface (SEF). L’exaltation de la chimiluminescence (SECL) est celle qui a été mise en évidence le plus récemment. Elle a été étudiée dans le cas de luminophores organiques au voisinage de particules ou de films rugueux d’or.

L’objectif de cette thèse consiste à développer deux systèmes de détection biologique :

- un système de double détection utilisant à la fois la résonance localisée de substrats métalliques et la luminescence de nanoparticules rendues luminescentes (chapitre IV),

- un système utilisant l’exaltation de chimiluminescence de luminophores à proximité d’une couche métallique rugueuse (chapitre V).

L’utilisation de substrats d’or est essentielle dans la réalisation de ces deux systèmes de détection biologique. Trois techniques permettent de préparer les substrats : ce sont les techniques de l’ablation par laser pulsé (PLD), de la lithographie utilisant des nanosphères comme masque (NSL) et de la lithographie par faisceau d’électrons (EBL). Dans le système d’ablation laser pulsé, lorsque un faisceau laser de très forte puissance arrive à la
surface de celle-ci, la cible s’évapore sous l’effet de l’absorption d’énergie du laser produisant ainsi un très grand nombre d’atomes et d’ions. Dès que les atomes et les ions de la cible gagnent le substrat, une couche mince peut se former. Plusieurs paramètres peuvent influencer les propriétés des couches minces. Dans ce mémoire on ne s’intéressera qu’à deux d’entre eux : l’épaisseur de la couche mince et la température du substrat. Lorsque l’épaisseur de la couche mince augmente, la couche mince passe de plate à rugueuse. Au contraire, lorsque la température de substrat augmente, la couche mince passe d’un état rugueux à plat.

La technique de lithographie par nanosphères permet de fabriquer des nanoréseaux d’or de la manière suivante. Une solution contenant des nanosphères de polystyrène est déposée sur un substrat de verre. Une seule couche ou une double couche de nanosphères s’organisent alors sur le substrat de verre en fonction de la température du dépôt. Une couche d’or est ensuite déposée sur la ou les couche(s) de nanosphères par ablation laser. Après décollement des nanosphères par ultrasons en présence de chloroforme, des nanoréseaux d’or apparaissent sur le substrat. La forme des nanoparticules élaborées à partir d’une seule couche de nanosphères est triangulaire alors que celle des nanoparticules élaborées à partir d’une double couche est hexagonale.

D’autres techniques permettant de préparer des nanoréseaux de particules d’or est la lithographie par faisceau d’électrons qui utilise un faisceau pour graver une couche de polymère (par exemple du PMMA) sur le substrat. Une couche d’aluminium est ensuite déposée sur la couche de polymère pour rendre le substrat conducteur. Après l’étape de gravure, la couche d’aluminium est dissoute grâce à une solution de potasse (KOH). Dès lors, l’échantillon est développé. Cette étape consiste en une dissolution des zones gravées du polymère possédant une masse moléculaire plus faible due aux brisures des chaînes lors de la gravure. L’étape suivante est l’évaporation d’une couche d’or sur le substrat. Enfin le polymère restant sur l’échantillon est retiré par un rinçage à l’acétone.

Cette méthode nous permet d’obtenir des nanoréseaux avec un pas variable supérieur à 200 nm.

Afin de réaliser une double détection, un objet luminescent peut également être attaché à la molécule biologique à détecter, sa luminescence pouvant être déclenchée par excitation UV, électrique ou chimique. Dans ce travail, des particules luminescentes nanohybrides seront utilisées. Ce sont des structures cœur/écorce composées d’un cœur à base d’oxyde de terre rare (luminescence inorganique) recouvert d’une écorce en polysiloxane contenant des molécules luminescentes (luminescence organique). C’est plus particulièrement l’oxyde de gadolinium dopé par des ions terbium (Gd$_2$O$_3$;Tb$^{3+}$) qui sera choisi pour former le cœur du nano-hybride. Ce choix est justifié par ses propriétés de transparence dans une large gamme de longueur d’onde avec une série d’émissions de 460 à 700nm de l’ion terbium.

Les particules de Gd$_2$O$_3$;Tb$^{3+}$ sont obtenues par la méthode polyol en partant d’une solution mixte de chlorure de gadolinium et de terbium comme précursors. Cette méthode a été mise au point dans l’équipe depuis plusieurs années (thèses de Rana Bazzi et de Marco Flores). Dans cette thèse, la synthèse initialement développée est optimisée et simplifiée. Au lieu d’ajouter la soude permettant la précipitation de l’oxyde à haute température (140°C), celle-ci est ajoutée dans la solution contenant les précursors à température ambiante. Nous avons ainsi montré que, contrairement à tous les résultats de la littérature qui indiquaient que le matériau ne se formait qu’à température élevée.
(au-dessus de 140°C), la synthèse des nanoparticules d’oxyde de terre rare pouvait se faire directement à température ambiante. Nous avons aussi mis en place un dispositif de suivi in situ de la synthèse par l’enregistrement simultané des spectres de luminescence. Nous avons alors montré que la vitesse d’addition de la soude est un paramètre crucial pour la croissance des grains d’oxyde. Une addition progressive de la soude permet d’obtenir dès la température ambiante des grains de plus grosse taille. Cette vitesse d’addition est aussi déterminante quant à la croissance ultérieure des particules si des traitements thermiques sont appliqués.

Afin de comprendre la structure électronique des nano-oxydes à l’échelle nanométrique, des particules de Gd₂O₃:Tb³⁺ ont été caractérisées par spectroscopie de perte d'énergie des électrons (EELS) qui permet de sonder les seuils d’ionisation des atomes. Cette technique permet bien sûr d’identifier la présence des éléments dans l'échantillon (elle en donne une signature chimique et en permet la quantification relative) mais elle permet également d’appréhender certains transferts d’électrons entre cations et anions qui pourraient se produire à l’échelle nanométrique. Nous avons ainsi montré que dans les spectres du seuil K de l’oxygène enregistrés pour des particules de tailles différentes, l’intensité et l’énergie des pics dépendent très fortement de la taille (un second pic apparaissant même pour les particules les plus petites) et que le spectre redevient caractéristique d’un matériau macroscopique au-delà de 2.7 nm. Nous avons interprété ce phénomène comme la signature d’une modification de la liaison chimique Gd-O en fonction de la taille des particules et avons été renforcés dans notre interprétation par des analyses fines des spectres au seuil N₄,₅ du Gd. Clairement, l’ensemble de ces résultats démontrent qu’un transfert d’électrons s’opère depuis le Gd jusqu’à l’atome O, sous l’effet d’une délocalisation d’une partie des électrons f par hybridation avec la bande de valence. Ce résultat généralise les observations menées sur le comportement des métaux (comme l’argent) à l’échelle nanométrique pour qui il a été montré qu’ils devenaient fortement réducteurs.

Les nanoparticules cœur/écorce Gd₂O₃/SiOₓ sont obtenues par enrobage des coeurs de Gd₂O₃ par un mélange d’organoalcoxysilanes, TEOS (tetraethyl orthosilicate) et APTES (aminopropyltriethoxysilane). Des fluorophores comme la fluoresceine isothiocyanate (FITC) ou la rhodamine B isothiocyanate (RITC) peuvent être conjugués à l’APTES et être ainsi incorporés à l’intérieur de la couche de polysiloxane. En plus de protéger le coeur contre l’eau, la couche de polysiloxane permet également la fonctionnalisation avec des effecteurs biologiques ainsi qu’une augmentation de la réponse optique du coeur luminescent du fait d’un transfert d’énergie entre le coeur et l’écorce. L’épaisseur de la couche de polysiloxane dépend de la quantité d’organoalcoxysilanes utilisée et du nombre de particules d’oxyde de gadolinium à enrober. Dans ce travail, des couches de polysiloxane d’épaississeurs différentes ont été déposées sur le même coeur de Gd₂O₃ pour comprendre les mécanismes de formation de cette couche de polysiloxane. Les résultats expérimentaux montrent que seulement une faible partie (10%-20%) de la solution d’enrobage a participé à l’élaboration de l’écorce de polysiloxane. La morphologie des particules de coeur/écorce est visualisée en imagerie en transmission électronique conventionnelle.

Afin d’effectuer la détection de la liaison entre des molécules de streptavidine et de biotine, des particules de Gd₂O₃@SiOₓ avec de la rhodamine B encapsulée sont fonctionnalisées avec des molécules de streptavidine selon une stratégie en deux étapes.
Premièrement, du p-phénylène diisothiocyanate est greffé sur les particules par la réaction entre une de ses fonctions isothiocyanate et les groupes aminés de surface. La deuxième fonction d’isothiocyanate qui reste libre permet de réagir dans un deuxième temps avec le groupe aminé de la streptavidine. A l’issue de ce processus, la streptavidine est alors liée de manière covalente à la particule. Ensuite, la streptavidine marquée par des particules de Gd₂O₃@SiOₓ est déposée sur des substrats constitués de nanoréseaux de plots d’or préalablement biotinyllés par des polypeptides greffés à une molécule de biotine. La fixation des particules sur le substrat d’or grâce au couplage entre la streptavidine et la biotine est vérifiée par la modification de la morphologie des plots d’or (lissés par le dépôt de particules) et l’augmentation de hauteur (de 7 nm) et de diamètre (de 16 nm) des plots d’or.

Le déplacement du pic SPR du substrat d’or est visualisé par la comparaison des spectres d’extinction qui montrent qu’il y a 3 nm de déplacement de ce pic après fixation de biotine sur le substrat et qu’il y a 21 nm de déplacement supplémentaire après la fixation de la molécule de streptavidine accrochée à la particule. Le résultat nous démontre que le pic LSPR peut être utilisé pour détecter la liaison streptavidine/biotine grâce au changement local de l’indice de réfraction du substrat quand les biomolécules sont fixées sur le substrat. L’étape suivante consiste à utiliser la technique de microscopie optique en champ proche (SNOM) comme méthode supplémentaire pour confirmer la liaison entre la streptavidine et la biotine. Le principe de cette technique de détection consiste à enregistrer les images optiques des nanoréseaux de plots d’or (luminescence) dans le but de détecter une différence après la fixation des nanoparticules luminescentes sur ces plots. Les images de SNOM montrent qu’il n’y a pas de signal optique sur le substrat d’or avant incubation de ce substrat avec des particules fonctionnalisées et luminescentes (RBITC) mais qu’il y en a un après. Les résultats obtenus démontrent donc qu’attacher une particule luminescente à de la streptavidine permet de détecter la liaison entre la streptavidine et la biotine de deux manières différentes : par le décalage du pic LSPR des substrats d’or d’une part et par la luminescence des nanoparticules fixées contenant des fluorophores encapsulés.

Le deuxième système pour détecter l’interaction entre la streptavidine et la biotine se fonde sur le phénomène d’exaltation de chimiluminescence par la surface (SECL) que nous avons été les premiers à mettre en évidence sur des couches. La chimiluminescence se produit sous l’effet d’une réaction chimique entre le luminophore (luminol) et le dioxyde d’hydrogène (H₂O₂) catalysée par la peroxydase. Afin de réaliser la détection, les polypeptides liés à des molécules de biotine sont fixés sur des substrats d’or fabriqués par la technique PLD grâce à la liaison entre les atomes de soufre du polypeptide et l’or. Ensuite, une solution contenant des molécules de streptavidine liées à des molécules de peroxydase est mise en incubation avec ces substrats d’or. Quand le substrat est plongé dans une solution contenant du luminol et H₂O₂, la réaction du luminol avec H₂O₂ est catalysée par la peroxydase fixée sur le substrat. Ainsi, les luminophores émettent une lumière à une longueur d’onde de 425 nm, ce qui permet de détecter que la liaison entre les molécules de biotine et de streptavidine s’est correctement opérée.

L’exaltation de chimiluminescence est démontrée à partir de l’étude de couches d’or fabriquées par PLD à des températures différentes. L’intensité de chimiluminescence augmente d’un ordre de grandeur quand la couche d’or est rugueuse par rapport à la valeur mesurée quand la couche d’or est plate. En outre, l’intensité de chimiluminescence
augmente légèrement en fonction de la température de fabrication des couches d’or, ce qui peut être corrélat à l’augmentation de leur rugosité. Un autre phénomène notable est que de l’émission par luminescence n’est observée qu’à l’endroit du substrat où de la peroxydase a été déposée, ce qui confirme de manière très claire que le rôle catalytique de la peroxydase est essentiel dans l’étape de luminescence.

Pour mieux comprendre l’exaltation de la chimiluminescence au voisinage de surfaces rugueuses, plusieurs paramètres ont été étudiés de manière systématique. Le premier d’entre eux est la distance entre le substrat et la peroxydase. Cette distance entre le substrat et la peroxydase est égale à la somme de la longueur du peptide biotinylé et du rayon de la molécule de streptavidine. Elle peut donc être modifiée en ajustant la longueur de ce peptide. Une chaîne de peptides est composée d’acides animés liés par des liaisons peptidiques. Dans notre travail, on a utilisé quatre sortes de peptide comportant 5, 11, 17 et 23 acides animés et faisant respectivement 1.3, 3.5, 5.7 et 7.8 nm de longueur.

Une première observation concerne les couches lisses d’or et d’alliage d’or et d’argent : l’intensité de la chimiluminescence augmente avec la distance substrat/peroxidase jusqu’à un plateau de 2000 en unités arbitraires du fait d’une diminution du quenching de luminescence par le métal. Selon la théorie du transfert de Föster, le quenching varie comme l’inverse de la puissance 4 de la distance entre le donneur (ici le luminol) et l’accepteur (ici le substrat métallique), ce qui est bien vérifié par nos expériences. Une deuxième observation concerne les couches rugueuses. Concernant ces dernières, l’intensité de chimiluminescence augmente avec la distance peroxydase/substrat quand celle-ci varie de 1.3 à 3.5nm puis diminue légèrement après cette valeur. Dans les mêmes unités arbitraires, la chimiluminescence arrive à un maximum de 30 000 pour une distance de 3.5nm. Comparée à la valeur de 2000 obtenue pour des substrats plats, la chimiluminescence est donc exaltée d’un ordre de grandeur. Nous avons démontré que cette exaltation est clairement due à la rugosité du substrat d’or et que le facteur d’exaltation diminue avec la distance du luminophore à celui-ci. Plus précisément, l’intensité de chimiluminescence est simultanément influencée par le quenching et l’exaltation. A courte distance (longueur de peptide inférieure à 3.5nm), le quenching est prédominant. Lorsque la distance augmente en revanche, c’est l’exaltation qui devient prépondérante. Cette double influence conduit à un maximum de chimiluminescence pour une longueur de peptide de 3.5 nm.

Le deuxième paramètre que nous avons étudié pour comprendre l’exaltation de la chimiluminescence est la nano-structure du substrat. Ainsi, outre les substrats plats et rugueux obtenus par PLD, des nanoréseaux de particules d’or fabriqués à partir d’une seule et ou d’une double couche de nanosphères (NSL) ont été utilisés dans cette étude. Après normalisation des intensités de chimiluminescence par l’aire effective des substrats, nous avons observé que la chimiluminescence au voisinage des réseaux de particules d’or est beaucoup plus importante qu’à proximité des couches plates et rugueuses. De la même manière, nous avons conclu que les réseaux élaborés à partir d’une double couche de nanosphères de latex exaltaient plus efficacement la luminescence que ceux fabriqués à partir d’une simple couche. Nous avons attribué ce phénomène au rayon de courbure particulier des plots d’or obtenus quand ceux-ci sont fabriqués à partir d’une double couche.

Le troisième paramètre auquel nous nous sommes intéressés est la valeur du pH de la solution contenant le luminol, H$_2$O$_2$ et la peroxydase. En fait, la réaction du luminophore
et H₂O₂ catalysée par la peroxydase comporte deux étapes. Dans la première étape, la peroxydase réagit avec H₂O₂ pour produire un complexe intermédiaire à base de peroxydase, ce complexe réagissant ensuite avec le luminol pour produire un deuxième type de complexe intermédiaire. Ce deuxième complexe intermédiaire peut alors produire l’oxydation du luminophore. Le pH favorisant cette étape est compris entre 8 et 9. Dans la deuxième étape, le luminol oxydé se transforme en 3-aminophthalate, espèce dans un état d’excitation qui, en revenant à l’état fondamental émet de la luminescence. Le pH favorisant cette deuxième étape est compris entre 10 et 11. Huit valeurs de pH entre 7 et 12 ont été choisies dans cette étude. Nous avons observé que la chimiluminescence au voisinage de couches rugueuses est toujours plus importante qu’à celui de couches plates et ceci quelle que soit la nature chimique de la couche (Au ou Ag) et quelle que soit la valeur du pH. De plus, le maximum de chimiluminescence est observé pour un pH de 9, une valeur qui favorise la catalyse de la réaction chimique par la peroxydase. Ceci constitue une première indication très précieuse que l’origine de l’exaltation pourrait être catalytique et non plasmonique.

Après avoir étudié les différents paramètres qui peuvent influencer la chimiluminescence, nous nous sommes attachés à étudier le mécanisme d’exaltation de celle-ci par la surface de substrat. Celui-ci est-il d’origine catalytique ou plasmonique ?

D’autres groupes de recherche ont démontré que l’intensité de chimiluminescence augmente avec l’intensité de la résonance plasmon et en ont logiquement conclu que l’exaltation de chimiluminescence, ainsi liée au recouvrement entre l’absorption plasmonique et l’émission du luminol, est un phénomène plasmonique. Dans ce mémoire, nous postulons qu’il n’en est rien. Nous faisons reposer cette affirmation sur la constatation qu’il y a bien une relation entre l’intensité de chimiluminescence et celle de la résonance plasmon du substrat mais que cette relation n’est pas universelle mais liée à la nature chimique du substrat. Or l’intensité du pic plasmon et celle de la chimiluminescence évoluent toutes deux dans le même sens avec la rugosité du substrat. Nous pensons donc que l’exaltation n’est pas produite par un mécanisme plasmonique mais par un mécanisme de catalyse et que la rugosité est le facteur déterminant de ce mécanisme.

Pour confirmer que le mécanisme d’exaltation est bien d’origine catalytique, nous avons étudié la chimiluminescence au voisinage de trois substrats rugueux de nature chimique différente (Au, Ag, Au-Ag) fabriqués à des températures différentes mais présentant tous un pic de résonance à une même position de 620 nm. L’intensité de ce pic est plus importante pour la couche d’argent alors que le phénomène d’exaltation est le plus faible pour cette couche. Cela confirme bien qu’il n’y a pas de relation directe entre l’intensité du pic de résonance et celui de chimiluminescence. Nous nous sommes également concentrés sur deux types de substrats d’argent (argent sur verre et argent sur BaTiO₃) qui présentent des pics de résonance à des longueurs d’onde différentes. La superposition entre la position du pic SPR et l’émission du luminophore (425nm) est plus importante pour l’argent déposé sur le verre que pour celui déposé sur BaTiO₃. Or les intensités de chimiluminescence présentent un rapport inversé, celle-ci étant plus importante pour l’argent déposé sur BaTiO₃ que sur le verre. Ces deux séries d’expériences confirment qu’il y pas de relation entre l’exaltation de chimiluminescence et la superposition entre la position du pic SPR et l’émission du luminol. Elles démontrent que le mécanisme d’exaltation de chimiluminescence ne saurait être d’origine...
Nous avons donc proposé des mécanismes catalytiques pour expliquer l’exaltation de chimiluminescence. Le mécanisme principal peut être déduit de l’apparente contradiction que les phénomènes catalytiques supposent des interactions à courte distance (inférieure au nanomètre) alors que la distance métal/peroxidase est ici beaucoup plus grande (5 nm). Puisque l’oxydation se produit en deux étapes, l’une impliquant la peroxidase et l’autre la présence d’un radical oxygène (non nécessairement à proximité de la peroxidase) c’est ce dernier radical qui est le plus probablement concerné par l’activité catalytique de l’or. Il serait donc logique que l’exaltation catalytique soit liée à un transfert d’électron des clusters métalliques à l’eau oxygénée adsorbée. Cela produirait des radicaux clés favorisant la formation des complexes, intermédiaires et radicaux intervenant dans la réaction chimique donnant lieu à luminescence. Le second mécanisme agirait de concert avec le premier. Le rayon de courbure des surfaces métalliques diminuerait le potentiel d’oxydoréduction du luminophore au voisinage de substrat d’or rugueux, favorisant ainsi l’oxydation du luminophore et exaltant de ce fait la luminescence émise.

En conclusion, deux systèmes de détection biologique ont été étudiés. Dans le premier système, nous avons fait la preuve qu’attacher une particule à une molécule à détecter permet une détection multimodale si la particule est luminescente. La fixation de la particule sur le substrat peut en effet être observée soit directement grâce à la luminescence de la particule soit par la modification des propriétés optiques du subtrat, celui-ci voyant sa résonance plasmon se décaler vers les grandes longueurs d’onde. Dans le deuxième système, la fixation de la streptavidine sur la biotine déposée sur le substrat d’or est détectée par chimiluminescence et nous avons Expliqué comment optimiser la structure du substrat (sa nature chimique, sa morphologie) ainsi que les conditions de l’expérience (le pH) pour exalter la chimiluminescence. Finalement, nous avons fait la preuve que cette exaltation tire son origine de phénomènes catalytiques et non plasmonique.
I Introduction
In the past two decades, the biotechnology and medical fields have seen great advances in the development of novel technologies that open new horizons for identifying and quantifying biomolecules and diagnosing diseases. These novel technologies consist of biosensor, biochip, microanalysis of biomolecules, molecular filtration, and sparse cell isolation, etc. Among these technologies, those related to biosensors and biochips are two extremely significant ones owing to their wide applications for the diagnosis and monitoring diseases, proteomics and the environmental detection of biological agents.[1]

The first studied biosensors were Surface Plasmon Resonance (SPR) sensors. The potential of SPR biosensors was recognized in 1980s by Liedberg et al.[2], who were able to detect immunoglobulin antibodies by observing the change in critical angle when the antibodies bind selectively on an Au film. Since their original discovery, SPR sensors changes have been used in refractive-index-based sensing to detect analyte binding at or near a metal surface. Moreover, the SPR sensors have been used to monitor a broad range of analyte-surface binding interactions as the adsorption of small molecules[3,4,5], protein adsorption on self-assembled monolayers[6,7], DNA hybridisation[8] and protein-DNA interactions[9].

Another technique used for the biodetection is the SERS (Surface Enhanced Raman Scattering). Since the 1980s, the phenomenon, that specific molecules at or near a roughed metal nanosurface undergo a big increase in the intensity of the inelastically scattered light, has led to an increasing number of biological applications[10] like the in vivo molecular imaging of cancer[11] or surface-enhanced Raman spectroscopy of DOPA-containing peptides[12]. Following the development of SERS, other surface-enhanced (SE) phenomena have been studied in the last decades like SE fluorescence (SEF) but SE chemiluminescence (SECL) is the last that has been evidenced.

Among diverse SPR and SERS biosensors and biochips, the ones based on nanostructure gold have attracted great deal of interests due to its two properties: the catalysis for chemical reactions and localized surface plasmon resonance. This thesis focuses two new biological detection systems to detect the binding of streptavidin/biotin:
- a SECL system based on chemiluminescence of luminol/hydrogen peroxide (H2O2) enhanced by gold nanostructures in the vicinity, which benefits from the catalytic property of nanostructure gold;
- a multimodal detection system using LSPR of the gold substrate and the luminescence of labelling core-shell Gd2O3/SiOx nanoparticles, which profits from the plasmonic property of nanostructure gold.

Chapter II gives the general introduction about two detection systems related to SPR, SECL, Au substrate and core-shell nanoparticles, etc. In chapter III, three techniques of preparing nanostructure thin film (gold, silver and alloy of gold and silver) are presented, which are pulsed laser depositions (PLD), nanosphere lithography (NSL) and electron beam lithography (EBL), respectively.

Following that, in chapter IV (concerning the multimodal detection system of LSPR and luminescence), core-shell nanoparticles (Gd2O3/SiOx) are successfully synthesized. Their structure are optimized by modified synthesis routes and visualized by transmission electron microscopy (TEM). Finally, the core-shell particles are demonstrated to be
successfully applied to a multimodal detection system using LSPR of substrate and the luminescence of labelling nanoparticles

In chapter V (concerning the SECL system), the phenomenon of SECL on the gold thin films is demonstrated. Aiming to better understand the correlative mechanism, SECL is optimized by the distance between peroxidase (catalyst for luminescence of luminol/\(\text{H}_2\text{O}_2\)) and thin films, the nanostructure of thin film, the pH value of luminol solution, and the chemical nature of thin films. At the end of this part, a catalytic effect is proposed to explicate SECL of luminol induced at the vicinity of corrugated gold film.

Finally, chapter VI gives the general conclusion obtained from the study of two detection systems.
References:

II General bibliography
In this part, we generalize the bibliography about the biosensor and biochip systems and the correlative methods of measurement. As important types of biosensor, SPR and LSPR biosensor were also introduced, as well as the ones based on SERS and SECL. Gold substrates and core-shell particles were finally presented owing to their significant role of increasing the sensitivity and selectivity of biosensor and the function as a luminescent tag allowed to be detected.

**Biosensor /biochip**

A biosensor is a device that consists of a biological recognition element or bioreceptor (e.g. an antibody, an enzyme, a protein, a nucleic acid, whole cells, tissues or whole organisms) and a signal transducer. When the analyte interacts with the bioreceptor, the resulting complex produces a change (e.g. conformation changes, etc.) which is converted into a measurable effect (e.g. an electrical signal) by the transducer. The most common types of bioreceptor/analyte complexes are based on: (1) antibody/antigen interactions, (2) nucleic acid interactions, (3) enzymatic interactions, (4) cellular interactions (e.g. microorganisms, proteins) and (5) interactions using biomimetic materials (e.g. synthetic bioreceptors). The most prevalent signal transduction methods include: (1) optical measurements (e.g. luminescence, absorption, surface plasmon resonance, etc.), (2) electrochemical (e.g. potentiometric, amperometric, etc.) and (3) mass-sensitive measurements (e.g. surface acoustic wave, microcantilever, microbalance, etc.).

Biochip is a special class of biosensors which has multiple transducer elements and is based on integrated circuit microchips. Being different with the original definition of term “biochip”, which comes from the word “computer chip” and is a silicon-based substrate used in the fabrication of miniaturized electronic circuit, a biochip is now generally defined as a material or a device that has an array of probes used for biochemical assays. In general, any device or component incorporating a two-dimensional array of reaction sites and having biological materials on a solid substrate has been referred to as a biochip.

Biosensors and biochips have been rapidly developed owing to different measurement methods. Optical measurements remain the most widely used signal transduction method for detecting biological binding event and for imaging in biological system. Various types of biosensors have been studied based on the optical measurements, for example, quantum dot in semiconductor used as a biological label, lanthanide nanohybrids encapsulating fluorescent molecules and porous silicon inducing optical interference.

Even though optical technique continues to evolve, the fact is that electrical detection remains extremely desirable. Electrical system can be miniaturized and integrated into systems, offering many advantages over optical detection schemes.
nanostructure, such as semiconductor nanowires\textsuperscript{[8,9]} and carbon nanotubes\textsuperscript{[10]} offer the greater chances for creating robust, sensitive, and selective electrical detectors of biological binding events. Current flow in any “one-dimension” system is extremely sensitive to minor perturbations, and in nanowires and nanotubes, the current flows extremely close to the surface. Biological macromolecules bound to the surface of a nanowire and undergoing a binding event with conformational change or change of charge state, may thus perturb the current flow in the nanowire. Thus, it is possible in principle that these materials will form the basis of new electrical biosensing systems, and important strides in this direction have been made.\textsuperscript{[2]} Figure II.1\textsuperscript{[11]} gives an example of field-effect transistors (FETs) nanowire biosensor which is based on electrically sensing result from the binding of a charged species to Si nanowire.

\textbf{Figure II.1 Nanowire FET sensor.} (A) Schematic of a regular planar FET device, where S, D, and G correspond to source, drain, and gate, respectively. (B) High-resolution
transmission electron microscopy image and electron diffraction pattern for a 4.5 nm diameter singlecrystal Si nanowire with <110> growth axis, and electrical transport data for a typical p-type nanowire that is characteristic of an FET. (C) Schematic of a Si nanowire-based FET device configured as a sensor with antibody receptors (green), where binding of a protein with net positive charge (red) yields a decrease in the conductance. (D) Cross-sectional diagram and scanning electron microscopy image of a single Si nanowire sensor device and a photograph of a prototype nanowire sensor biochip with integrated microfluidic sample delivery.

More complex physical behaviour, beyond quantum-confined semiconductor systems (single-electron-like behaviour), metals (with collective plasmon excitations), or even ordinary electrical devices, arises in systems with correlated electron behaviour, including nanoscale magnetic systems. The physics of small magnetic systems was first discussed decades ago, and is undergoing a renaissance as it becomes possible to study magnetic phenomena in individual nanoparticles and even individual molecules. Magnetic crystals behave as a single magnetic domain, with all the spins in a very small crystal, and at a high enough temperature, this moment wanders randomly (superparamagnetic); above a critical size, this moment becomes locked in a fixed direction (ferromagnetic). Magnetic nanocrystals are widely employed in artificial biological detection and separation systems, serving important roles as magnetic resonance contrast enhancement agents and as the basis for a wide range of magnetophoresis experiments.

**SPR and LSPR biosensor**

Among diverse biosensors developed for diagnosis and monitoring diseases, proteomics and the environmental detection of biological agents, the first studied ones were Surface Plasmon Resonance (SPR) sensors. The potential of SPR biosensors was recognized in 1980s by Liedberg et al. who were able to detect immunoglobulin antibodies by observing the change in critical angle when the antibodies bind selectively on an Au film. Since their original discovery, SPR sensors changes have been used in refractive-index-based sensing to detect analyte binding at or near a metal surface. Moreover, the SPR sensors has been used to monitor a broad range of analyte-surface binding interactions as the adsorption of small molecules, protein adsorption on self-assembled monolayers, DNA hybridisation and protein-DNA interactions. Figure II.2 illustrates a SPR-based biosensor which provides a gold-coated surface through which minute changes in biomolecule concentration. These changes lead to changes in the reflected light that are captured by the optical detection unit and converted to a resonance signal.
As an optical biosensor technique that measures molecular binding events at a metal surface by detecting changes in the local refractive index, SPR offers several advantages over conventional techniques such as fluorescence or ELISA (enzyme-linked immunosorbent assay). \cite{29} First, because the measurements are based on refractive index changes, detection of an analyte is label free and direct. The analyte does not require any special characteristics (scattering bands) or labels (radioactive or fluorescent) and can be detected directly, without the need for multistep detection protocols (sandwich assay). Second, the measurements can be performed in real time, allowing the user to collect kinetic data, as well as thermodynamic data. Last, SPR is a versatile technique, capable of detecting analytes over a wide range of molecular weights and binding affinities. \cite{30} Because of its unique features, SPR has become a powerful tool for studying biomolecular interactions.

Besides SPR sensors, Localized Surface Plasmon Resonance (LSPR) sensors are also extremely interesting for different groups, since they induce a local refractive index changes after adsorption of biological molecules. \cite{31,32,33,34,35,36,37,38} The differences between the SPR and the LSPR sensors are respectively their refractive index sensitivities 2.106 nm/RIU (Refractive Index Unit) \cite{39} and 2.102 nm/RIU \cite{31} and respectively their evanescent electric field

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**Figure II.2 (Left)** Schematic presentation of SPR-based biosensor. When the solution of the analyte ("ligand") flows the surface of gold film ("acceptor"), the interaction between the ligand and the acceptor an increased quantity of material is fixed on the surface, the quantity being proportional to the refractive index of light. Detection is possible because the resonance angle changes with the refractive index. The shadows in the reflex indicate the loss of energy which can be detected by the SPR-based instrument. (Right up) The resonance angle changes from I to II with the change of molecule quantity on the surface of biosensor. (Right down) The resonance angle monitored in real-time augments with the increase of quantity of the complex between the ligand and the acceptor.
decay lengths $l_d = 200-300$ nm\cite{39} and $l_d = 5-30$ nm\cite{31,32,33}. However, the two types of sensors are very competitive in their sensitivities, because the two characteristics are compensated. The LSPR nanosensors require no temperature control (low refractive index sensitivity) compared to the SPR nanosensors where a temperature control is necessary because of the large refractive index sensitivity.

**SERS and SECL**

Another important application based on the surface plasmon phenomena is surface-enhanced Raman Spectroscopy (SERS)\cite{40,41}. Raman spectroscopy is a vibrational spectroscopic method that has the inherent ability to distinguish between molecules with great similarity, such as the structural isomers glucose and fructose\cite{42}. Unfortunately, high laser powers and long acquisition times are usually required to achieve high quality Raman spectra due to the inherently small normal Raman scattering (NRS) cross-section of many molecules of interest\cite{43}. Higher intensity Raman signals and lower detection limits can be achieved using SERS. SERS produces very large enhancements in the effective Raman cross-section of species spatially confined within zone of the electromagnetic fields (0–4 nm)\cite{44} generated upon excitation of the LSPR of nanostructured noble metal surfaces. This large electromagnetic field induces a dipole in nearby molecules, thus enhancing Raman scattering from absorbed molecules.\cite{45}

The effects of SERS are composed of two contributive origins, the electromagnetic enhancement\cite{46} and the chemical enhancement.\cite{47} Electromagnetic enhancement results from the enhancement of local electromagnetic fields at the surface of a metal which can support surface plasma/optical conduction resonances. Chemical enhancement is associated with the charge transfer between the metal and adsorbate at atomicscale roughness features.\cite{48}

In comparison with other spectroscopies such as infrared absorption and normal Raman scattering spectroscopies, SERS enjoys the advantages of application in aqueous media and the sensitivity sufficient for trace level detection.\cite{49} This enables SERS spectroscopy to be one of the most effective trace analytical methods. Although SERS intensity varies from sample to sample due to the substrate morphology, by choosing an appropriate internal standard or developing a large calibration data set, SERS can be used for quantitative detection. The distinct advantages of SERS, such as low detection limit, real-time response, both qualitative and quantitative analysis capabilities, a high degree of specificity, and simultaneous multi-component detection, make it applicable in identification and characterization of pharmaceuticals,\cite{50} bacteria,\cite{51} and other molecular species.\cite{52} Moreover, owing to the phenomenon that specific molecules at or near a roughed metal nanosurface undergo a big increase in the intensity of the observed inelastically scattered light, since the 1980s, SERS offers an increasing number of biological application\cite{53} like the in vivo
molecular imaging of cancer\textsuperscript{[54]} or surface-enhanced Raman spectroscopy of DOPA-containing peptides\textsuperscript{[55]}. In Figure II.3\textsuperscript{[56]}, an application of SERS for cancer diagnosis is given, in which molecules near the nanorods on the cancer cells are found to give a Raman spectrum that is greatly enhanced (due to the high surface plasmon field of the nanorod assembly in which their extended surface plasmon fields overlap), sharp (due to a homogeneous environment), and polarized (due to anisotropic alignments).

![Figure II.3 Left: SEM images of normal and cancer cells; right: Raman spectra of (A) normal cells (B) cancer cells.](image)

Following the development of SERS, other surface-enhanced (SE) phenomena have been studied in the last decades like SE fluorescence (SEF),\textsuperscript{[57]} whereas SE chemiluminescence (SECL) is the last one that has been evidenced. Chemiluminescence, which is the phenomenon observed when the vibronically excited product of an exothermic reaction relaxes to its ground state with emission of photons, can be defined in simplistic terms: chemical reactions that emit light.\textsuperscript{[58]} The chemical reaction produces energy in sufficient amount (approximately 300 kJ mol\textsuperscript{-1} for blue light emission and 150 kJ mol\textsuperscript{-1} for red light emission) to induce the transition of an electron from its ground state to an excited electronic state. This electronic transition is often accompanied by vibrational and rotational changes in the molecule. In organic molecules, transitions from a $\pi$ bonding to a $\pi^*$ anti-bonding orbital ($\pi \rightarrow \pi^*$) or from a non-bonding to an anti-bonding orbital ($n \rightarrow \pi^*$) are most frequently
encountered. Return of the electron to the ground state with emission of a photon is thus called chemiluminescence.\textsuperscript{[59]}

The attractiveness of chemiluminescence as an analytical tool lies primarily in the simplicity of detection; the fact that most samples have no unwanted background luminescence, as is typically observed in fluorescence-based assays; and the fact that no optical filters are required to separate the excitation wavelengths and scatter, as is also required for fluorescence-based detection.\textsuperscript{[60]} Recently, CL has been reported to be enhanced near gold\textsuperscript{[61]} and silver\textsuperscript{[62]} particles or corrugated films\textsuperscript{[63]}. However, the mechanisms responsible for this SECL remain still controversial and, similar to SERS, could be related to either a plasmonic enhancement or a catalytic effect. A series of papers prove that, contrarily to SEF, a catalytic effect is more possible than a plasmonic one. This observation is consistent with the general observation that, even if bulk gold is chemically inert, gold particles turns out to be surprisingly active for many reactions\textsuperscript{[64,65,66].}

**Au substrates**

Whatever LSPR or SECL, the role of nanostructured gold is important. Biosensors and biochips of highly sensitivity and selectivity have been greatly improved by the development of gold nanoparticles and thin films. In its bulk form, gold has been regarded to be chemically inert towards chemisorption of reactive molecules such as oxygen and hydrogen. Consequently, pure gold was considered to be an uninteresting metal from the point of view of catalysis. The most noticeable exception to this was its use as a ‘diluent’ for an active metal: the addition of the inert gold to an active metal such as platinum affects to a significant extent the selectivity of the catalyst.\textsuperscript{[67]} However, recently, gold catalysts have attracted a dramatic growth of interest, since gold was reported to be extremely active for catalyzing a large range of chemical reactions if deposited as nanoparticles on partly reducible oxides.\textsuperscript{[68]}

Moreover, Gold nanoparticles as direct or indirect probe are applied to achieve efficient electrical, colorimetric or surface enhanced Raman scattering biological detection.\textsuperscript{[69]} The SERS effect has been extensively studied in gold nanostructures, which produce remarkably amplified electromagnetic field of the incident light by localized surface plasmon resonance. The enhancement factor of SERS using gold nanoparticles is estimated to be $10^6$–$10^{15}$,\textsuperscript{[70]} and it is extremely dependent on the size and shape of nanoparticles. This is due to the existence of so-called “hot spots” having intense electromagnetic fields in which highly efficient Raman scattering can be obtained, for example, a junction of the adjacent two particles, protrusions of particles such as in the rod-like nanoparticles (nanorods). As shown in Figure II.4,\textsuperscript{[71]} Au nanosphere array substrate was used to substantially improve the sensitivity and reproducibility of SERS measurement for a novel immunoassay, which exploits the SERS-derived signal from reporter molecules (crystal violet, CV) encapsulated in
antibody-modified liposome particles. The concentration of the antigen was found to be indirectly read out by the SERS intensity of the CVs.

![Schematic diagram of the immunoassay based on SERS at gold nanosphere array substrate.](image)

Figure II.4  Schematic diagram of the immunoassay based on SERS at gold nanosphere array substrate.

Gold thin films are also widely used as chip substrates or electrodes to immobilize organic molecules via the helpful Au-S covalent bond.\[72,73\] For example, the SPR of gold thin films was used to detect the dielectric constant changes induced by the molecular adsorption at the surface of the films.\[74\] Gold films could further be used to design devices liable to detect the specific adsorption of molecules by the luminescence of labelling dyes, even if dye fluorescence is found to be quenched in contact with metallic beads.\[75,76\] This quenching is due to the re-absorption of the dye emission by the gold surface plasmon according to a resonant energy transfer process.\[77\] However, luminescence of luminol brought to the vicinity of gold films was proved to be enhanced instead of quenched by roughness in some optimized condition.\[63\] 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.\[78\]
Core-shell nanoparticles

Besides the biosensors based on LSPR or SECL, another possibility of biological detection has been particularly studied, which consists in attaching a tag to the molecule that can be detected under further UV, electrical or chemical excitation.\textsuperscript{[79]} Luminescent particles, especially hybrid (core-shell) particles consisting of core covered by shells of different chemical composition can be chosen as the tag.

Core-shell particles have been attracting a great deal of interest due to their fantastic properties, different from those of single-component materials. Coating the particles with a thin shell of a compatible material makes it possible to control the interparticle and particle–matrix interactions, thereby further improving functional properties and expanding a broader range of potential application. The structure, size, and composition of these particles can be altered easily in a controllable way to tailor their magnetic, optical, mechanical, thermal, electrical, electrooptical, or catalytic properties.\textsuperscript{[80]}

It has been reported that core-shell particles (Fe\textsubscript{3}O\textsubscript{4}/Eu:Gd\textsubscript{2}O\textsubscript{3}) (Figure II.5\textsuperscript{[81]}) could be used for DNA quantification in a hybridization-in-solution approach; that Fe/Au core-shell structured nanoparticles may be used in biological applications as magnetic resonance imaging (MRI) agents, cell tagging and sorting and targeted drug delivery;\textsuperscript{[82]} that Gd\textsubscript{2}O\textsubscript{3}:Eu\textsuperscript{3+}@SiO\textsubscript{2} core-shell particles (Figure II.6\textsuperscript{[83]}) possessed great potential in quantum phosphor applications; and that Gd\textsubscript{2}O\textsubscript{3}/SiO\textsubscript{x} can be applied as multimodal contrast agents for in vivo imaging.\textsuperscript{[84]}

\textit{Figure II.5 TEM image of core-shell particles (Fe\textsubscript{3}O\textsubscript{4}/Eu:Gd\textsubscript{2}O\textsubscript{3}).}
Figure II.6  (a) SEM photograph of Gd$_2$O$_3$:Eu$^{3+}$ thin film coated on the surface of silica particles and (b) BSE (Back Scattered Electron) -SEM photograph.

Luminescent core-shell nanoparticles with a Gd$_2$O$_3$ core encapsulated within a polysiloxane shell (Gd$_2$O$_3$@SiOx) (Figure II.7) can also be bound as labels for molecules (streptavidin) to detect after grafting onto Au particles arrays. The choice to bind a particle (and not a single dye) to the molecule to detect lies on two reasons. First, the high hindrance of particles allows expecting a higher LSPR shift (this was already proved for deposition of metal clusters and CdTe quantum dots on corrugated gold films with a random roughness). Second, particles are generally brighter than single luminophore whatever they consist of quantum dots with high intensity emission or form a platform incorporating multiple dyes. As such, these nanobeads emit more bright light than single fluorophore, are more stable against photobleaching and do not suffer from intermittent on/off light emission. Core-shell nanoparticles Gd$_2$O$_3$@SiOx functioning together with Au particles arrays allow developing multimodal sensors by detecting intrinsic luminescence and substrate LSPR shift.
simultaneously.
References:


[80] G. Liu, G. Hong, D. Sun, J. Colloid Interface Sci., 2004, 278, 133..


III Preparation of metal thin films
In this chapter, two techniques were presented to fabricate nano-structure substrates. Bulk-like and cluster-like metal thin film (Au, Ag, and AuAg alloy) can be prepared by PLD technique by controlling two main parameters: substrate temperature and film thickness. Gold particle arrays can be obtained by lithography techniques (NSL and EBL). The morphologies of obtained nano-structure substrates were shown by AFM images and their optical properties were investigated by extinction spectra.

### III.1 Pulsed Laser Deposition (PLD) technique

#### III.1.1 Presentation of PLD technique

The fabricating film technique of pulsed laser deposition first appeared on the 1960s. Then the scientists found that a luminescent and plasmonic area that was made of solid particles would be formed at the vicinity of solid surface, when the laser reacted with the solid. If these particles in plasmonic state sprayed outside and deposited on the substrate, thin film would be formed on the substrate. That was the process of film fabrication by laser.\(^1\) In 1965, Smith et al firstly tried to fabricate the optical films with laser,\(^2\) whereas at that time the method of vapor deposition was dominant and PLD technique just was found and was not perfect, which caused that the technique was not developed greatly in the subsequent decades. The situation was not changed until the middle of 1980s. In 1987, Dijkkamp and X.D. Wu utilized the technique of laser evaporation to obtain superconducting films, which inspired the great interest of researchers in superconducting research.\(^3\) The technique of fabricating films with the laser was rapidly developed, and the high Tc superconducting films, ferroelectric thin films, optical and Photoelectric Films, semiconductor, metal and Superhard Material films were successfully fabricated by the technique. Now the technique of laser becomes the main technique for preparation of thin films.
Typical PLD system is showed in Figure III.1. It is mainly made up of vacuum chamber, heater, target, substrate and inflatable system, etc. When a bouquet of laser with certain power density arrive at target surface, the target surface will be instantly melted and evaporated due to the absorption of high energy of laser, and produce a mass of plasma that contain atoms and ions, which is called as laser plume. According to the principle of Momentum Conservation, laser plume will move along the direction perpendicular to the target surface. When laser plume arrive at the substrate, the deposition of films can be achieved in certain favourable conditions.

In the process of film preparation by PLD technique, many parameters are needed to be optimized since they influence the film’s growth quality and their physical properties. Firstly among the parameters, laser’s energy density, wavelength, pulse-width, and repetition frequency are the most important as they influence directly laser plume’s component and size, so they influence the films’ component and the preparation’s speed; secondly the target’s components determines the films’ components ,so the target has an important effect on film preparation. More compact the target, much less the particles and cavities on the films. Besides the laser and target, the pressure in vacuum chamber and the temperature of substrate are also two key parameters during the preparation of films. The pressure effects the interaction between laser and target, the laser plume’s size, form and spreading speed, the speed of film deposition, and films’ physical properties. In the process of film preparation, the substrate is placed on a heater, such as silicon heater or resistance wire heater, to real-time control the temperature of films. The formation of films is a reconstruction process of many molecules, atoms and ions, so it is closely related to these particles’ kinetic energies and
diffusion speeds, which makes the temperature become the very important parameter. Furthermore, the distance between the target and the substrate, the choice of substrate and the annealing after deposition etc, all have the affect on the films’ components, structures and physical properties. This shows that films preparation is a complex growth process and is limited by many factors. Only all of these parameters are studied and optimized, the good quality films will be obtained.

### III.1.2 Preparation of thin films by PLD technique

#### III.1.2.1 Preparation of Au thin films

The foil of Au (purity 99.99%, diameter 40mm) chosen as target was placed from the quartz substrate about 40mm, and a XeCl excimer laser [308 nm, 17 ns full width at half maximum] operating at 1Hz repetition rate is focused onto the target in vacuum (~10Pa). All the experimental conditions were displayed in Table III.1.

*Table III.1 PLD experimental conditions of Au thin films preparation at different substrate temperature*

<table>
<thead>
<tr>
<th>Target</th>
<th>Au foil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>quartz</td>
</tr>
<tr>
<td>Excimer laser</td>
<td>XeCl,308nm</td>
</tr>
<tr>
<td>Laser pulse width</td>
<td>20ns</td>
</tr>
<tr>
<td>Laser energy density</td>
<td>~2J/cm²</td>
</tr>
<tr>
<td>Laser Frequency</td>
<td>1Hz</td>
</tr>
<tr>
<td>Number of Laser pulses</td>
<td>2400</td>
</tr>
<tr>
<td>Substrate temperature</td>
<td>variable</td>
</tr>
<tr>
<td>Deposition gas</td>
<td>10Pa (N₂)</td>
</tr>
</tbody>
</table>

If the number of Laser pulses was fixed (2400), an Au film with thickness of 25nm was able to be obtained. As showed in Figure III.2, at room temperature Au thin film presented bulk-like characters without surface plasmon resonance (SPR) peak in the absorption spectra.
However with the substrate temperature increasing (above 200°C), the SPR peak appeared in the absorption spectra, and Au films transferred from bulk-like material to cluster-like material, and SPR band shift blue accordingly.

![Absorption spectra of Au thin films prepared at different substrate temperature](image)

**Figure III.2** Absorption spectra of Au thin films prepared at different substrate temperature

![AFM images (area of 500 nm x 500 nm) of Au thin films prepared at 30°C (a), and 500°C (b) respectively.](image)

**Figure III.3** AFM images (area of 500 nm x 500 nm) of Au thin films prepared at 30°C (a), and 500°C (b) respectively.

From the AFM images (**Figure III.3**), we observed that Au thin film prepared at room temperature (30°C) was continuous and flat, whereas with the temperature increasing, Au films become rough because of the appearance of nanoparticles. The roughness of Au thin films prepared at 30°C, 200°C, 300°C, 400°C, 500°C and 600°C was 0.3, 1.3, 1.5, 1.5, 1.6
and 1.9, respectively. So the roughness of Au thin films increases with the substrate temperature increasing.

As we know, the SPR peaks are determined by many factors. Here it is the presence of clusters on the Au thin films prepared at high substrate temperature who give rise to SPR peaks at the range of visible light. The change of SPR peak position and FWHM came from the change of nano-microstructure on the surface of Au thin film. A possible explanation is as following: the variety of particle size on the surface of Au thin film can be divided into two parts: one is on the x-y plan; the other is in the Z axis. When the substrate temperature increased, the size change of nanoparticles in the Z axis augmented, which caused the surface roughness of Au thin film augmented; whereas the decrease of the size change in X-Y plan is the reason for the shift blue of SPR peaks and the decrease of peak width.

III.1.2.2 Preparation of Ag thin films

On the basis of preparation of Au thin films, Ag films also were prepared by PLD technique. The foil of Ag (purity 99.99%, diameter 30mm) chosen as target was placed from the quartz substrate about 40mm, and a XeCl excimer laser [308 nm, 17 ns full width at half maximum] operating at 1Hz repetition rate is focused onto the target in vacuum (~10Pa). Ag thin films were obtained at different substrate temperature and the absorption spectrum were displayed in Figure III.4.

As shown in Figure III.4, Ag thin films presented the same absorption properties as the Au thin films: the films transformed from bulk-like characteristic to cluster-like one, which was proved by the appearance of SPR peak; the changes of SPR peak position of Ag thin films were in agreement with that of Au thin films: blue shift with the increase of substrate temperature. The AFM Images of Ag films prepared in room temperature and 500°C are shown in Figure III.5.
III.2.3 Preparation of Au-Ag thin films

Besides Au and Ag films, Au-Ag alloy films also were fabricated by PLD technique at different substrate temperature. A disk target that can rotate in plane was made of Au and Ag fan-shaped foils, the Au/Ag ratio of alloy films can be controlled easily by adjusting their fan-shaped area ratio. Here, the alloy films of Au/Au ration about 1:1 were prepared at different substrate temperature. The quartz which was chosen as substrate was placed from the foil of alloy about 40mm, and a XeCl excimer laser [308 nm, 17 ns full width at half maximum] operating at 4~6Hz repetition rate is focused onto the target in vacuum (~10Pa).

Figure III.6 indicated the absorption spectrum of Au-Ag thin films prepared at different substrate temperature. Au-Ag thin films prepared at room temperature displayed the same characteristic as Au thin films prepared at room temperature; when temperature was increased,
Au-Ag thin films presented SPR peaks like the Au and Ag films, as well as the SPR peak shifted to blue and the FWHM reduced. In general, the SPR position of Au-Ag thin films located between those of Au and Ag films. The AFM Images of Ag films prepared in room temperature and 500°C are shown in Figure III.7, which present the similar morphology as Au and Ag films.

![Figure III.6 Absorption spectra of Au-Ag alloy thin films prepared at different substrate temperature.](image)

![Figure III.7 AFM images (area of 500 nm×500 nm) of Au-Ag alloy thin films prepared at 30°C (a), and 500°C (b) respectively.](image)

All the results above indicated that SPR peak position can be adjusted by controlling the experimental conditions. Except for the substrate temperature, there are many factors to vary the characteristics of metal thin films, such as alloy ratio, annealing, transition layer, gas pressure, and deposition speed, etc. For instance, the lower the air pressure during the PLD deposition, the flatter the surface of thin film; the lower the deposition speed, the smaller the FWHM of SPR peak. After being annealed, the SPR peak of thin film prepared at high
temperature will shift blue, and the bulk-like film prepared at room temperature will turn to cluster-like film. The presence of transition layer of BaTiO$_3$ will enhance adhesion between metal surface and quartz substrate.

**III.2 Nanosphere Lithography (NSL) technique**

**III.2.1 Presentation of NSL technique**

Nanosphere lithography is an inexpensive, simple and high throughout nanofabrication technique. As shown in *Figure III.8*, the process of fabrication mainly consists of three steps: NSL mask preparation, film deposition and nanospheres liftoff.

![Nanosphere lithographic fabrication](image)

*Figure III.8* Nanosphere lithographic fabrication of nanoparticle arrays and film over nanosphere surfaces.

A. NSL mask preparation

The NSL masks are created by self-assembly of polystyrene nanospheres mixed in the water. The ordered 2D colloidal crystals are formed during the process of evaporation of solvent where polystyrene nanospheres self-assembled owing to capillarity between adjacent nanospheres: a grain of crystallization can be formed firstly, then nanospheres around gradually move close to the grain of crystallization during the evaporation, finally a single layer (SL) or double-layer (DL) mask as shown in *Figure III.9* can be obtained.
Various methods have been used to prepare NSL masks, among which the simplest one is to dropcoat a suspension of polystyrene nanospheres onto glass substrate where nanosphere self-assembled into hexagonally close-packed 2D colloidal crystal. Whereas by this method, it is to difficult to obtain large areas arrays and the nanospheres used are limited to be smaller than 600 nm in diameter. So other methods such as spin-coating and continuous convective assembling[11] have been utilized to optimize the evaporation rate and grain growth rate, thereby to improve quality of nanosphere and reduce defects of 2D crystals. In our work, a modified method based on these principles was used to obtain large areas arrays of 2D colloidal crystal in an order of cm².

B. film deposition

Within 2D crystals formed by periodic particle arrays, interspaces between adjacent nanoparticles are also regular arrays so as to be serviced as the template of nanoparticle arrays. When a layer of material is deposited onto the nanoparticle masks, the interspaces in the masks will be filled with the materials. Nanoparticle arrays can be formed as soon as nanosphere masks are lifted off. A monolayer mask produces nanoparticles having a triangular inplane shape arranged on the surface with p6mm symmetry (Figure III.9C), and a

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**Figure III.9** Schematic diagrams of single-layer (SL) and double-layer (DL) nanosphere masks and the corresponding periodic particle array (PPA). (A) a (111) SL mask, dotted line=unit cell, a=first layer nanosphere; (B) a(111)p(1 ×1)-b DL mask, dotted line=unit cell, b=second layer nanosphere; (C) SL PPA, 2 particles per unit cell; (D) DL PPA, 1 particle per unit cell.
double-layer mask makes the same hexagonal arrays with p6mm symmetry but nanoparticles have a hexagonal inplane shape (Figure III.9D).

A variety of materials, such as noble metal, magnetic materials, semiconductor materials, polymers, and protein can be used as deposition materials in NSL. The methods of deposition consist of physical vapour deposition, electron beam deposition, and pulsed laser deposition, etc. materials are usually vertically deposited onto the mask, or else the shape and distance of nanoparticles can be varied with angles of deposition.

C. nanospheres liftoff

After deposition of materials, the polystyrene nanospheres can be removed from substrates by dissolving them in organic solvent with the aid of sonication. The size and arrangement of nanospheres determine structures of nanoparticle arrays. Figure III.10 gives the arrays obtained from monolayer and double layer nanospheres. Lattice parameters for two types of arrays can be deduced from their geometry of the masks.

For monolayer mask array (Figure III.10 (a)), distances between two particles are given by

\[ a_1 = \frac{D}{\sqrt{3}} , \]
\[ a_2 = D , \]
\[ a_3 = \frac{2D}{\sqrt{3}} \]

where D is the diameter of nanosphere. The in-plane particles are triangle and the size parameters are given by:

![Figure III.10 Schematic diagrams of nanoparticle arrays prepared by single-layer (SL) and double-layer (DL) nanosphere masks.](image-url)
\[ h_{SL} = \frac{3}{2}(\sqrt{3} - 1 - \frac{1}{\sqrt{3}})D = 0.233D \]

\[ b_{SL} = 2h_{SL} / \sqrt{3} \]

For double-layer mask array (Figure III.10 b), distances between two particles are given by

\[ a_1 = D \]

\[ a_2 = \sqrt{3}D \]

The in-plane particles are hexagonal and the size parameters are given by:

\[ h_{DL} = (\sqrt{3} - 1 - \frac{1}{\sqrt{3}})D = 0.155D \]

\[ b_{DL} = h_{DL} / \sqrt{3} \]

### III.2.2 Preparation of gold dot arrays by NSL

Firstly, a mask consisting of a single or a double-layer of polystyrene nanospheres was deposited on glass substrates by a self-assembly process. Briefly, 30 µL of diluted nanosphere solution (1wt% solid, diameter of 200 nm) was dropped onto a cleaned glass substrate that was inclined between 4º and 6º in a chamber with saturated humidity. By controlling the temperature between 35ºC and 45ºC, 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.
Figure III.11 AFM images of the gold nanoparticle arrays elaborated by NSL: (a) 2D and (b) 3D images of arrays by using a monolayer sphere mask, (c) 2D and (d) 3D images of arrays by using a double layer sphere mask.

**Figure III.11** (a) and **Figure III.11** (b) gives the 2D and 3D AFM image of particle arrays fabricated using a single-layer nanosphere mask (SL Au), respectively. They show a well-ordered hexagonal array belonging to the p6mm plane group. Each cell contains 2 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). **Figure III.11** (c) and **Figure III.11** (d) exhibit the arrays fabricated using a double layer nanosphere mask (DL Au). The hexagonal array belongs to the same p6mm 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, a localized surface plasmon resonance (LSPR) corresponding to a coherent oscillation of the spatially confined electrons is induced. 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. 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 conditions results in a very low efficiency of the non-resonant light-to-SPP coupling so that, for the randomly corrugated films studied in this letter, only LSPR should be considered. For particles arrays, the metal dots are deposited on a non-conducting quartz substrate. Thus SPP can not propagate and LSPR is again the only plasmon to consider. As shown in Figure III.12, 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 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.

![Figure III.12 Extinction spectra of nanoparticle arrays fabricated with monolayer (SL Au17, 24, and 33) or double layer (DL 18) nanosphere masks.](image)

Figure III.12 Extinction spectra of nanoparticle arrays fabricated with monolayer (SL Au17, 24, and 33) or double layer (DL 18) nanosphere masks.
III.3 Electron Beam Lithography (EBL) technique

III.3.1 Presentation of EBL technique

Au dot arrays used as substrates for a double detection were fabricated by EBL, a technique permitting to control precisely the shape and the size of the metallic nanoparticles, also the distance between the nanoparticles and consequently permitting to tune the local surface plasmon resonance (LSPR) of metallic nanoparticles arrays. \cite{15,16} EBL is an alternative to the nonosphere lithography (NSL) to design nanosensors with an efficiency similar to those designed by Van Duyne and co-workers.\cite{17} The main physical difference with the NSL technique is that EBL permits a precise control of the shape of the metallic nanoparticles and a great variety of shapes. Moreover, EBL allows to fabricate monodisperse systems of particles with simple and controlled shapes, thus it becomes possible (easier with nanocylinder than with NSL technique) to calculate the number of molecules adsorbed with a relatively good precision and thus to give a value of more accurate sensitivity.

![Schematic representation of EBL process.](image)

The preparation process of Au dot arrays by EBL is displayed in Figure III.13. Firstly, glass substrates are cleaned in a freshly prepared piranha solution (3:1 H\textsubscript{2}SO\textsubscript{4} (98%), H\textsubscript{2}O\textsubscript{2} (30%)) for 30 min. Once cooled, the glass substrates are rinsed abundantly with deionized water and dried with N\textsubscript{2} gas. Then a layer of about 150 nm of poly (methylmethacrylate)
(PMMA: 950 k) is deposited by spin coating (4000 rpm) on glass substrates and then baked during 20 min at 160°C. Afterwards, an aluminum layer of 10 nm is evaporated on the PMMA in order to make the substrate conductive. The desired pattern is obtained by EBL using a Hitachi S-3500 SEM associated with nanometer pattern generation system (NPGS) from Nabity. The layer of Al is removed using a potash solution (KOH: C = 0.2 M during 30 s). The development of the exposed regions is performed with a mixture of methylisobutylketone (MIBK) and isopropanol (ISO) (1:3 MIBK/ISO) for 60 s. The masks are then rinsed in isopropanol for 10 s. After gold evaporation, a nanocylinders array can be obtained via a lift-off process dipping in acetone during 3 h.

III.3.2 Preparation of gold dot arrays by EBL

Au dot arrays fabricated by EBL permit to be used as substrates for the double detection. As shown in Figure III.14, which gives the morphology of the arrays evidenced respectively using SEM (Figure III.14 (a), (c)) and AFM (Figure III.14 (b), (d)), square arrays belong to p4mm

![SEM and AFM images of Au particles](image_url)

*Figure III.14 SEM (a) and AFM (b) images of Au particles separated by a centre in centre distance of 340 nm; SEM (c) and AFM (d) images of Au particles separated by a centre in centre distance of 1140 nm.*
plane group in the nomenclature of the International Tables for Crystallography (primitive cell, one fourfold rotation point, two mirrors). Each primitive cell contains one gold dot with a height of \( h = 70 \) nm and a diameter of \( d = 140 \) nm. These values were optimized to obtain the greatest sensitivity to further adsorption of biological molecules. Also their LSPR presents a slight overlap with dye emission (emission wavelength of Rhodamine: \( \lambda_{\text{emission}} \approx 590 \) nm) avoiding then a strong luminescence quenching of the dyes. Two types of samples differing by the centre in centre distance between gold dots (lattice parameter) were prepared: dots separated by 1140 nm (Figure III.14 (c), (d)) were used for obtaining optical images; dots separated by 340 nm (Figure III.14 (a), (b)) were studied in LSPR measurements (the large quantity of gold leading to high extinction). In the arrays so elaborated, the individual plasmon resonances are influenced by electromagnetic dot interactions. Near-field coupling which is only significant for nearly touching dots (separated by a distance of some tens of nm \[19\]) should be negligible. On the contrary, far-field dipole-dipole coupling between the individual dots is expected to significantly contribute to the extinction spectra.\[20\]

### III.4 Conclusions

In this chapter, three techniques (PLD, NSL and EBL) were used to fabricate the nano-structured metal substrates. Bulk-like and cluster-like thin films (Au, Ag, and Au-Ag alloys) were prepared by PLD technique by controlling two main parameters: the substrate temperature and the film thickness during deposition. The substrates become rough from flat with the increase of substrate temperature. On the contrary, the substrates become flat from rough with the increase of film thickness. Gold nanoparticle arrays were prepared by NSL using a single layer or double-layer polystyrene nanosphere mask. The obtained gold arrays show a well-ordered hexagonal array belonging to the p6mm plane group. The shape of the particles is consistent with that of the interstices let by the polystyrene nanospheres i.e. triangular and hexagonal for SL Au and DL Au arrays respectively. Two SPR 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 anisotropy of particles. Another method of lithography fabricating gold dot arrays is EBL, which permits to serially obtain patterns with shape, size, and interparticle space. The obtained square arrays belong to p4mm plane group, in which each primitive cell contains one gold dot with a height of \( h = 70 \) nm and a diameter of \( d = 140 \) nm. Two types of samples differing by the centre in centre distance between gold dots were prepared: dots separated by 1140 nm were used for obtaining optical images; dots separated by 340 nm were studied in LSPR measurements (the large quantity of gold leading to high extinction).
Reference:


IV Double detection by shift of SPR peak and luminescence of nanoparticle
A multimodal detection system was investigated in this chapter. It is based on the shift of SPR peak of gold substrate and the luminescence of core-shell nanoparticles encapsulating fluorophores. Tb-doped Gd$_2$O$_3$ core particles were synthesized by a polyol method which was optimized by following up the in-situ luminescence, and the electrical structure of core particles was characterized by EELS. A polysiloxane shell was successfully coated to the core particles and the core-shell structure was visualized by TEM images. After fixation of gold substrates (dot arrays or rough films), the core-shell particles can be used to detect the streptavidin-biotin binding by both the shift of the SPR peak of the substrate and the optical signal of the particles.

IV.1 Nanoparticle of core-shell Gd$_2$O$_3$@SiO$_x$

IV.1.1 Core of Gd$_2$O$_3$:Tb$^{3+}$

IV.1.1.1 Presentation of nanoparticle of core Gd$_2$O$_3$:Tb$^{3+}$

Lanthanide elements belong to the family of rare earth, including 15 elements from lanthanum (La) to lutetium (Lu). Lanthanide luminescence detection has been established as a powerful approach in bioassays in recent years. Lanthanide oxides are commonly used as luminescent materials in the lighting industry. Nanoparticles of lanthanide oxides have large Stokes shifts, narrow line-shaped emission bands, and long-lived luminescence (approximately 1–2 ms). They also have inherent photostability. In contrast with semiconductor quantum dots, the emission wavelength of the oxide nanoparticles is independent of particle size and hence monodispersity is less crucial, leading to lower synthesis costs. Surface modification does not, furthermore, significantly affect their optical properties, because their luminescence arises as a result of electronic transitions of the lanthanide ion.$^{[1]}$

Lanthanide ions are good candidates for luminescent centers due to their special 4f intra-shell transitions. Particularly, trivalent terbium ions ($4f^8$) are widely used as luminescent activator for a considerable number of phosphors because of its narrow green
bands originating from intra 4f-transitions. Under excitation at 280nm they present a series of emission ranging from 460 to 700nm relative to the transitions between the $^5D_4$ and $^7D_n$ (n=0-6) levels. Gadolinium oxide, which is easily obtained due to abundant resource in nature, has been widely used as host material owing to its refractory property; capacity of substitution and transparence in a large range of wavelength. When terbium ions are doped to gadolinium oxide, energy transitions shown in Figure IV.1 occurs in the nanoparticles Gd$_2$O$_3$:Tb$^{3+}$, then luminescenc of Tb$^{3+}$ can be enhanced.

![Figure IV.1 Energy transitions in nanoparticle Gd$_2$O$_3$:Tb$^{3+}$](image)

**Figure IV.1 Energy transitions in nanoparticle Gd$_2$O$_3$:Tb$^{3+}$**

IV.1.1.2 Optimization of nanoparticle of core Gd$_2$O$_3$:Tb$^{3+}$ by following up the in-situ luminescence

In our previous works, Tb$^{3+}$-doped Gd$_2$O$_3$ particles have been prepared by the polyol method. In these typical preparations, the mixed solution of precursor (chloride or nitrate of lanthanides) and doping element were heated and stirred at 140°C for complete dissolution of the components, and then aqueous NaOH was added to form the lanthanide oxide.

The classic synthesis route is optimized and simplified by adding NaOH at room temperature in the mixed solution of GdCl$_3$ and TbCl$_3$ (Tb is chosen as the doping element because of its narrow green emission bands originating from intra 4f-transition).
demonstrate, by following-up and observing the *in-situ* luminescence spectra of Gd$_2$O$_3$:Tb$^{3+}$ that the terbium doped gadolinium oxide can be formed at room temperature as soon as NaOH is added in the solution. The speed of addition of NaOH is also a crucial parameter for the growth of gadolinium oxide particles. We prove that a progressive addition of NaOH benefits to the growth of oxide particles. In particular, after heat treatment bigger sizes of Gd$_2$O$_3$:Tb$^{3+}$ particles can be obtained which is favorable to the optical properties of lanthanide particles.

The detailed synthesis process is described as following: Rare-earth chlorides GdCl$_3$ and TbCl$_3$ (99.9% Aldrich) were added in 250ml diethylene glycol (Merck 99%) to obtain a precursor solution at a total lanthanide ions concentration of 150 mM in the stoichiometric Tb/Gd ratio of 5/95. After being strongly stirred for 30 min, aqueous NaOH (3M) was added to the mixture in a quantity leading to a reaction yield of 30%. This addition was made according two different methods. In the first method, aqueous NaOH was added *at once* at room temperature instead of being added at 140°C as usual. \[3,4,7\] In the second method, aqueous NaOH was added at room temperature progressively: precisely 1/10 of the total quantity of NaOH was added step by step every minute. After NaOH addition, the mixed solution was kept at room temperature for 1h and heated to 160°C for 4h under vigorous stirring in refluxing DEG. As a result, transparent suspensions of particles dispersed in organic solvent were obtained. The resulting oxide particle suspensions were colloidal stable for years. The synthesis was followed up by observing, *in-situ*, the luminescence spectra for getting information on the formation and growth of particles in real time.

*Figure IV.2 Schematic representation of synthesis of Tb$^{3+}$-doped Gd$_2$O$_3$ by following up *in-situ* luminescence.*
Optical spectra were obtained as displayed in Figure IV.2 by using a 150-W Xe lamp as the excitation source. For emission spectra, excitation was adjusted at 275 nm (resolution 4nm) with a Jobin Yvon GEMINI double monochromator. Light was collected with an optical fiber coupled to a Jobin Yvon TRIAX320 monochromator and a CCD camera. A filter was added to avoid the second order of the monochromator. For excitation spectra, the wavelength was selected between 200 and 400nm using a Jobin Yvon GEMINI double monochromator. The fluorescence light was collected through the optical fiber, passed through a high-wavelength-pass filter (λ>480 nm) and detected by a photomultiplier (EMI9789). All measurements were carried out at room temperature. The excitation spectra presented are not corrected from the wavelength dependence of the excitation source, but they still are convective when being compared among themselves.

Figure IV.3 Excitation (a) and emission (b) spectra of Gd$_2$O$_3$:Tb$^{3+}$.

Figure IV.3 (a) shows the excitation spectrum of Gd$_2$O$_3$:Tb$^{3+}$ nanometer-sized particles monitored at an emission wavelength of 545nm. These particles were prepared according the polyl method described above and annealed at 160°C for 4 hours. Below 240nm the increase in the excitation spectra is ascribed to the valence to conduction band absorption of gadolinium oxide. The excitation peaks from wavelength 278 to 315nm (except the one at 280nm) are evidences of the Gd$^{3+}$ → Tb$^{3+}$ energy transfer due to good matching of excited state 4f levels of Gd$^{3+}$ and Tb$^{3+}$ ions.$^{[8]}$ They correspond to the gadolinium transitions from $^8S_{7/2} \rightarrow ^6I_j$ (J=7/2, 9/2, 11/2…) for the broad line around 278 nm and from $^8S_{7/2} \rightarrow ^6P_{3/2,5/2,7/2}$ for that around 315
The two ions form a coupled system so that energies absorbed by Gd$^{3+}$ are transferred to Tb$^{3+}$ by a non-radiative mechanism and are then emitted as Tb$^{3+}$ fluorescence.\[9\] The energy transfer doesn’t occur until Gd$^{3+}$ and Tb$^{3+}$ ions are close enough, therefore their appearance is indicative of the constitution of systems containing both atoms. Below these peaks appears a broad band which corresponds to the inter configurational transitions $4f^n \rightarrow 4f^{n-1}5d$ from Tb$^{3+}$ in the Gd$_2$O$_3$ matrix. As a result, the presence of this band, as well as that of the band to band transition, can be considered as an proof of the formation of Tb$^{3+}$ doped Gd$_2$O$_3$ during the synthesis. The peak at 285 nm and those between 340 and 390 nm originate from the 4f intra configuration of Tb$^{3+}$ ions regardless they are in solution or in the solid phase. \textbf{Figure IV.3}(b) displays the typical emission spectrum excited at 275 nm of Gd$_2$O$_3$:Tb$^{3+}$. It is in agreement with those previously published.\[2, 10\] Four peaks from 470 nm to 640 nm correspond to $^5D_4 \rightarrow ^7F_{6,5,4,3}$ transition of Tb$^{3+}$. The most intense peak of $^5D_4 \rightarrow ^7F_5$ is located at about 545 nm and three other peaks are observed at 485 nm ($^5D_4 \rightarrow ^7F_6$), 585 nm ($^5D_4 \rightarrow ^7F_4$) and 620 nm ($^5D_4 \rightarrow ^7F_3$). The spectrum indicates that luminescent centers were efficiently formed during the elaboration process.

\textbf{Figure IV.4} Excitation spectra of Gd$^{3+}$ and Tb$^{3+}$ solution before and after one-off adding NaOH at room temperature. Insert: TEM image of Gd$_2$O$_3$:Tb$^{3+}$ after adding NaOH at room temperature.

\textbf{Figure IV.4}, the excitation spectra for the precursor solution of Gd$^{3+}$ and Tb$^{3+}$ before and after a rapid (on-off) addition of NaOH at room temperature. Before NaOH addition, the band to band and the intensities of the peaks between 278 and 315 nm (corresponding mainly to
gadolinium transition and being then indicators of the formation of Gd$_2$O$_3$:Tb$^{3+}$) are small compared to those that are present between 340 and 390nm. However, as soon as NaOH is added in the solution at room temperature, the intensities of these peaks strongly increase and become 1.5 times higher than the ones characteristic of Tb$^{3+}$ 4f intra-transitions. The intensity enhancement of these peaks indicates that a terbium doped gadolinium oxide is formed after NaOH addition at room temperature. This result was unexpected because precipitation using the polylol method was only observed at high temperatures regardless the type of the materials elaborated, sulfides,$^{[11]}$ elemental metals$^{[12,13]}$ or oxides.$^{[14]}$ In particular, these lanthanide oxides were only prepared between 140 and 180°C until now.$^{[3]}$ The size distribution obtained by photon correlation spectroscopy (PCS, see Appendix) and a TEM image of the so-formed Gd$_2$O$_3$:Tb$^{3+}$ particles are shown in Figure IV.8 (a). Particles size is about 1.1nm with a mean standard deviation of 0.4 nm regardless the technique used. It is much smaller than that obtained for usual elaboration between 140 and 180°C (leading to a size ranging between 2 and 4 nm). This could be explained by a greater preservation of the capping properties of the solvent at low temperature. Additional EDX analysis performed with a spot size of 1 nm has shown that each particle contains Gd and Tb in the expected proportions so that solid solutions are, as for elaboration at high temperatures,$^{[3]}$obtained at room temperature. This is in agreement with the luminescence measurements of Figure IV.3 and Figure IV.4 that evidenced an energy transfer between the two lanthanides Gd and Tb.

Figure IV.5 displays the evolution of excitation spectra when NaOH is progressively added in the precursor solution at room temperature. With progressive addition of NaOH, the peaks between 278 and 315nm and the band to band absorption gradually increase, which is an indication that Gd$_2$O$_3$:Tb$^{3+}$ particles are gradually formed during the process of NaOH addition. In the same time, the peaks between 340 and 390nm originating from the 4f intra configuration of Tb$^{3+}$ ions remain unchanged. Compared with the spectra of a rapid (on-off) NaOH addition (Figure IV.4), all the peaks in Figure IV.5 are slightly better defined than those in Figure IV.4 which could indicate a better crystallization or a larger size of the particles formed. The corresponding size distribution and TEM image are shown in Figure IV.8(c). The size of the particles is about 1.3 nm with a mean standard deviation of 0.5 nm and is slightly bigger than that obtained for a rapid NaOH addition. This could result from a decreased nucleation rate which is, according to LaMer theories, $^{[15]}$ dependent on the concentration of reacting ions and then diminished when NaOH is added progressively. It is worthy to note that the particles are arranged in rods as it is shown by the inset of Figure IV.8(c). Each rod consists of an alignment over approximately twenty nanometers of particles joined together along planes which possess an inter-reticular distance of 3.1 Å. The cell parameter of cubic Gd$_2$O$_3$ is 10.81 Å, these planes correspond then to the (222) ones. For bcc structures (Ia3), the denser direction is the [111] one and the less dense planes are the (222)
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Such planes possess then a high surface energy which indicates that the formation of particles boundaries along (222) faces within the rods is certainly surface-energy driven. One can also notice that the (110) faces are neutral (no excess of charges) while the (222) faces are not.\textsuperscript{[16]} Thus the (222) faces are not expected to be as stable as they are in the NaCl or CsCl structures which is an additional reason for such an organization.

![Excitation spectra of Gd\textsuperscript{3+} and Tb\textsuperscript{3+} solution before and after progressively adding NaOH at room temperature. Insert: TEM image of Gd\textsubscript{2}O\textsubscript{3}:Tb\textsuperscript{3+} after progressively adding NaOH at room temperature.](image)

Figure IV.5  Excitation spectra of Gd\textsuperscript{3+} and Tb\textsuperscript{3+} solution before and after progressively adding NaOH at room temperature. Insert: TEM image of Gd\textsubscript{2}O\textsubscript{3}:Tb\textsuperscript{3+} after progressively adding NaOH at room temperature.

Annealing at high temperature (at 160°C) the so-formed particles is beneficial to their growth. This is proved by the excitation spectra in Figure IV.6. Figure IV.6(a) shows the evolution of the excitation spectrum of Gd\textsubscript{2}O\textsubscript{3}:Tb\textsuperscript{3+} with temperature at a heating rate of 1°C/min in the case of particles formed by a rapid (on-off) addition of NaOH. The spectra are normalized at the same arbitrary value for the peak at 352 nm corresponding to 4f intra configuration of Tb\textsuperscript{3+}. Both the band to band absorption below 240 nm and the peak related to the Gd\textsuperscript{3+}→Tb\textsuperscript{3+} energy transfer at 315 nm are significantly increased compared to the peaks originating from the 4f intra configuration of Tb\textsuperscript{3+} ions. In particular, the valence band to conduction band absorptions (below 240 nm) slightly increases in intensity from 0.3 to 0.6 (in arbitrary units). This result can be explained by the greater size of the particles evidenced by both TEM image and PCS spectra shown in Figure IV.8 (b). Indeed, the size of the Tb doped Gd\textsubscript{2}O\textsubscript{3} particles slightly increases from 1.1 nm (mean standard deviation of 0.4 nm) to 1.3 nm (mean standard deviation of 0.5 nm) during the annealing.
In the case of a progressive addition of NaOH (Figure IV.6 (b)), the evolution of the normalized excitation spectrum with temperature is similar to that observed in the case of the rapid one. The most obvious difference is that the enhancement of the Gd$_2$O$_3$ matrix absorption below 240 nm is now more pronounced. Concretely, the normalized intensity of the gap shoulder at 240 nm increases from 0.3 to 1 when the temperature is increased from 30°C to 160°C leading to an intensity enhancement of 0.7 which is more than two times greater than that obtained (0.3) in the case of a rapid addition. This result must be correlated to, on one hand, an increased crystallinity of the particles as evidenced by the TEM images of Figure IV.8(d) and on the other hand to a morphology change and to a large increase of the size of Gd$_2$O$_3$:Tb$^{3+}$ particles confirmed both by TEM image and PCS (Figure IV.8(d)). After annealing, Gd$_2$O$_3$:Tb$^{3+}$ particles are almost spherical (polyhedrons with a low aspect ratio) and have a size of 3.5 nm with a mean standard deviation of 1.1 nm. It is very striking that the volume of the cylindrical rods before annealing (between 20 and 25 nm$^3$) exactly coincides to that of the particles after annealing (23 nm$^3$). This would imply that during annealing diffusion occurred within the rod to re-organize the atoms in polyhedrons such as rhombic dodecahedrons limited by (110) faces that possess a low energy.

**Figure IV.6** Excitation spectra of Gd$^{3+}$ and Tb$^{3+}$ solution during increasing the temperature for (a) one-off adding NaOH (b) progressively adding NaOH; (c) and (d) are the spectra of (a) and (d) before the normalization of the intensity, respectively.
Finally Figure IV.7 compares the normalized excitation spectra recorded at room temperature before and after annealing for (a) a rapid and (b) a progressive NaOH addition. First, one can notice that in both cases (rapid or progressive addition) the spectra before and after annealing are very similar and differ only by the band to band peak at 240 nm that significantly increases during annealing. This is explained by the increased crystallization evidenced by TEM that enhances the absorption of the gadolinium oxide host and the related transfer to the Tb\(^{3+}\) doping ions. On the contrary, the excitation peaks related to the Gd\(^{3+}\)\(\rightarrow\)Tb\(^{3+}\) energy transfer between 278 and 315nm do not undergo any modification during annealing. This indicates that the energy transfer between Gd and Tb is fully efficient as soon as a condensed phase containing both cations is formed and does not depend on the characteristics of this phase. In particular it is not favored by a greater crystallization or a size increase of the gadolinium oxide host. This could indicate that the energy levels of both lanthanides are only slightly modified by the nature of the phase formed, Gd\(^{3+}\) and Tb\(^{3+}\) ions presenting a same efficient matching of excited state 4\(f\) levels before and after annealing.

Figure IV.7 Excitation spectra of Gd\(^{3+}\) and Tb\(^{3+}\) solution at room temperature before and after annealing for (a) one-off adding NaOH (b) progressively adding NaOH;(c) and (d)
are the spectra of (a) and (d) before the normalization of the intensity, respectively.

By comparison between spectra of Figure IV.6 and Figure IV.7, it is also obvious that the normalized intensity of the peak located at 315 nm and related to an energy transfer between Gd and Tb depends on the temperature. More generally we systematically observed that the intensity of this peak increases with temperature but has the same value upon heating or cooling. This is the proof that this peak intensity does not depend on the nature of the crystal formed but only on the temperature. To follow an in-situ structural or morphological evolution of the oxide synthesized, only the valence band to conduction band absorption at 240 nm is then relevant.

![TEM images](image)

**Figure IV.8**. TEM image of Gd$_2$O$_3$:Tb$^{3+}$ particles obtained by one-off adding NaOH (a) before (b) after the heat treatment at 180°C and by progressively adding NaOH (c) before (d) after the heat treatment. The insets were the size distribution of corresponding Gd$_2$O$_3$:Tb$^{3+}$ particles.
IV.1.1.3 Characterisation of nanoparticle of core Gd$_2$O$_3$:Tb$^{3+}$ by EELS

The physical properties of nanoparticles (smaller than 10 nm) differ strongly from those of bulk material especially when their size is small. Two different reasons explain this particular behavior. First, in the size-regime where the wavelength of the electrons is of the same order than the particle itself, quantum size effects can appear.$^{[17]}$ Second when the size decreases, surface becomes predominant relatively to the bulk so that the thermodynamics properties of the whole particle (including surface and bulk ones) are modified.$^{[18]}$ This leads for instance to grain-size driven phase transitions (for example, silver melting point is decreased by 700°C)$^{[19]}$ and to a modification of the electrochemical properties of the material.$^{[20]}$ The standard redox potential of the nanoparticle shifts negatively from that of the corresponding bulk provided that the surface free energy is the same for both.$^{[21]}$ In the case of silver, the monomer Ag is a strong electron-donating reagent which can reduce many inorganic and organic compounds$^{[22]}$ and clusters undergo electron loss compared to bulk. In this thesis, we aim at showing that this phenomenon is not limited to metals but can be also found in gadolinium oxide, an electron transfer occurring from Gd to O on the nanometer scale.

IV.1.1.3.1 Presentation of EELS technique

![Figure IV.9 System of EELS spectrometer in TEM](image)

Electron energy-loss spectroscopy (EELS) in the TEM involves the measurement of the
energy imparted to a thin ($\leq$200nm) specimen by fast ($\geq$100keV) incident electrons. There are number of ways in which the incident electrons interact with the specimen, giving rise to various features in the energy loss spectrum. One of the most important energy-loss processes is atomic ionization, in which electrons are ejected from inner, or core, shells (K, L, M, etc.) of atoms in the specimen. This process requires that the core electron receives an energy greater than or equal to the critical ionization energy, which is a function of the specific atom and the specific electron shell and is therefore uniquely defined. The characteristic signals termed ‘ionization edges’ appear in the spectrum at energy losses corresponding to the critical ionization energy, thus identifying the presence of specific elements in the specimen. The edge intensity can be related directly and quantitatively to the amount of the element present.

Conventional TEM has been fitted with electron spectrometer, and the latter is placed below the TEM viewing chamber as shown in Figure IV.9. The object of spectrometer is an image or diffraction of specimen which is at the image plan of project lens. The electron beam enters the spectrometer via the entrance aperture and is aligned by loops to be able to enter the prism. The smaller the entrance aperture is, the higher the spectrum’s energy resolution is. The prism disperses the electrons according to different electrons’ energy. The interference from exterior electric and magnetic field is insulated by electrically isolated drift-tube. The following quadrupole lenses are used to magnify the energy-loss information, and the attenuator can deflect the electrons with strong energy from YAG scintillator surface in order to protect photodiodes. Finally, the spectra can be obtained by CCD camera.

![Figure IV.10 Typical EELS spectrum](image)

The typical electron energy-loss spectrum is displayed in Figure IV.10. It consists of
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zero-loss peak, plasmon peak, ionization edge (or core loss edges, including edges of energy-loss near-edge structure (ELNES) and edges of extended energy-loss fine structure (EXELFS). The zero-loss peak represents the elastic scattering signal; the plasmon peak arises from outer shell inelastic scattering of plasmon; the ionization edge is a result of removal of electrons from the core of the atom resulting in energy losses. ELNES results from small intensity fluctuation of ionization edge within 50eV of the threshold, whereas EXELFS occurs beyond about 50eV above the ionization threshold.

IV.1.3.2 Characterization of core Gd$_2$O$_3$:Tb$^{3+}$ by EELS

Four types of Gd$_2$O$_3$:Tb$^{3+}$ nanoparticles with different sizes were characterized by EELS to understand the electronic structure of the nanoparticles modified with the size. The sizes of particles are 1.1nm, 1.5nm, 2.7nm and 4nm with a mean standard deviation of 0.4, 0.5, 0.9 and 1.5 nm, respectively. For all samples, the size distribution determined by Photon Correlation Spectroscopy (PCS) is given in Figure IV.11 and confirmed by the high resolution Transmission Electron Microscopy (TEM) images shown in insets.

![Figure IV.11](image-url)

**Figure IV.11** Size distribution determined by photon correlation spectroscopy of Gd$_2$O$_3$ nanoparticles: (a) 1.1nm (b) 1.5nm (c) 2.7nm (d) 4nm. The insets show the HRTEM images of correspondent particles.

The Gd$_2$O$_3$ samples were prepared by deposing a drop of a diluted colloidal solution on a holey carbon grid. EELS was carried out in a TEM-Image Coupled configuration using a
JEOL 2010F microscope operating at 200kV and equipped with a Gatan Digipeels 766 spectrometer. The probe size was of the order of 80 nm. In all experiments, the energy resolution derived from the zero loss peak (ZLP) full width at half maximum (FWHM) was equal to 0.9 eV. Two series of experiments were successively achieved with a resolution of 0.1 and 0.3 eV/channel, respectively. The resolution of 0.1 eV allowed to obtain EELS spectra with high resolution. The lower resolution permitted to locate the absolute positions of the different edges relatively to the C K one at 283.8 eV. All the measurements were performed at room temperature. After acquisition, the spectra were corrected from the spectrometer dark current and pixel to pixel gain variations of the detector array. They were de-convoluted from the zero-loss spectrum using a Fourier ratio technique given by the PEELS software.

![EELS spectra of GdO₃:Tb³⁺ nanoparticles](image)

**Figure IV.12** EELS (a) oxygen K edge and (b) gadolinium N₄,5 edges of GdO₃:Tb³⁺ nanoparticles with different sizes.

Spectra obtained at the oxygen K and gadolinium N₄,5 edges are shown in **Figure IV.12** (a) and (b) respectively. In **Figure IV.12** (a), the four O K spectra recorded for the four different sizes are given and compared to the multiple scattering simulation of Botton *et al.* obtained
for cubic Gd$_2$O$_3$ (grey curve). A clear evolution can be observed when the size increases from 1.1 nm to 2.7 nm. For the bigger particles, only one peak is observed (peak B) whereas for the smaller ones, two peaks (A and B) are present. This (or these) peak(s) are followed by a smooth bump lying around 535 eV. Peak B is located at exactly the same position (540.8 eV) regardless the size whereas the intensity and the energy of peak A strongly increase when the size decreases. For the bigger particles, the spectra dominated by peak B are in agreement with the simulation obtained for cubic Gd$_2$O$_3$ which indicates that the structure of gadolinium oxide is cubic. For the smallest particles, the modification of the spectra is not compatible with a cubic $\rightarrow$ monoclinic transition since this latter should induce a shift of the peak B towards high energy (here the peak B remains at the same energy and the peak A locates at lower energy) and a sharpening of peak B (here the peak is broadened). High resolution TEM confirms that the cubic phase is stable regardless the size since a same inter-reticular (222) distance of 3.1 Å is observed for all the samples. Such a distance is in perfect agreement with the cell parameter of the Ia3 gadolinium oxide: 10.82 Å. The conclusion is unambiguous since there is a difference of 2% between the (222) planes of the Ia3 cubic lattice and the (111) planes of the C2/m monoclinic structure whereas the precision of our high resolution TEM is less than 1% on reticular distances. Also the luminescence spectra of Eu introduced as a doping element (5%at) in gadolinium oxide confirm that this latter is cubic for all sizes. As a consequence of all these observations, the cubic $\rightarrow$ monoclinic transition observed for Gd$_2$O$_3$ particles prepared by Low Energy Cluster Beam Deposition (LECBD) and maintained in a clean and controlled environment below 2.8 nm is not obtained when the particles are prepared by the polyol method. This is explainable by the difference of surface energies between both particles. The surface energy of the particles prepared by LECBD is of a solid/gas type and then significantly higher than that (of a solid/liquid type) of the particles prepared by the polyol method which are strongly capped by the solvent. The cubic $\rightarrow$ monoclinic transition being surface-driven, it should arise at a smaller size for particles prepared chemically. To give an order of magnitude, the critical size is decreased by at least a factor of ten for barium titanate and iron oxide when the surface shifts from a solid/gas type (ceramics) towards a solid/solid type (powder). Since the O K edge can essentially be considered as a one electron process (direct transitions from O 1s to O p levels), it is closely related to the empty O p Density O f States (DOS). In rare earth oxides, these states are mainly mixed with the rare earth 5d states and, to a lesser extent, with the 6s and 4f levels, to form the valence and conduction bands. The changes observed in Figure IV.12 (a) are thus a signature of the modification of the Gd-O chemical bond as a function of the particles size. Such changes only occur for very small particles and bulk properties are recovered for sizes above 2.7 nm.

To go deeper in the understanding of these effects, we have focussed on the Gd N$_{4,5}$ edges.
These are given for each particle size in Figure IV.12 (b) and compared to the bulk reference of Ahn et al. (grey curve). No significant chemical shift was observed and all the spectra show a peak at 145.3 nm. This edge is very different from the O K one since it is dominated by multiplet effects arising from the strong overlap between the Gd 4d and localized 4f/ radial wave functions. These effects give rise to the narrow pre-edge peaks below 140 eV (arrowed in Figure IV.12(b) and magnified in the inset) and the broad structure around 145 eV. X-ray Photon Electron Spectroscopy (XPS) experiments, X-ray Absorption Spectroscopy (XAS) experiments and calculations on various rare earth (RE) oxides showed that these 4d-multiplet structures, and especially the pre-edge ones, are very sensitive to the solid-state hybridization between the RE 4f and O 2p states. Careful examination of the inset reveals that these structures are indeed broader for the 1.1 and 1.5 nm particles and shifted by 0.5 eV towards higher energies compared to those obtained for the 2.7 and 4 nm particles or the bulk reference. In agreement with the changes observed at the O K edge, it is found again that size induced effects are only sensitive for particles below 2.7 nm. An equivalent broadening was reported when comparing the 4d-XAS of Ce in its α and γ phase and was attributed to an increase of the hybridization strength between the Ce 4f states and the valence band. In our case, this broadening goes with an energy blue shift of 0.5 eV. This energy is comparable to the V parameter used in calculations to account for the solid-state hybridization between the RE and O atoms (V = 0.76 eV in CeO₂ and 0.56 eV in Pr₂O₃). This energy shift can be linked to changes in the exchange integrals between Gd 4d and 4f/ wave functions arising from the delocalization of the Gd 4f electrons. The observed changes can thus be directly correlated to the delocalization of a small part the Gd 4f electrons via their hybridization with the Gd 5d and 6s states which results in their mixing with the O 2p states. This delocalization mechanism was proposed from first principal calculations by Strange et al. in 1999 in order to understand the valence of rare earths in various compounds.

[36]
The DOS of cubic Gd$_2$O$_3$ can be schematized as presented in Figure IV.13 (Gd-DOS in grey, O-DOS in black). Concerning the oxygen atom, only the O 1s and $p$ levels are given since we are focusing on the O K edge. From bottom to top of the energy scale (left panel), one finds the deep O 1s core-levels, the Gd 4$d$ levels and the Gd 4$f$ states which, according to several XPS measurements$^{[37,38]}$, are found at the bottom of the valence band. The valence band is formed by the hybridization of the O 2$p$ with the Gd 5$d$ and 6$s$ states. Above the Fermi level is the conduction band formed by the anti-bonding states which are probed by EELS. Following the self-interaction-corrected local-spin-density calculations of Petit et al. performed on a hexagonal Gd$_2$O$_3$ structure$^{[39]}$ and in agreement with assumptions made by Strange et al.$^{[35]}$the unoccupied Gd 4$f$ states are at the bottom of this conduction band while the unoccupied Gd 4$d$ and 6$s$ states are higher in energy. The proportion of the O $p$-DOS in these bands is directly correlated to the mixing of the O 2$p$, Gd 4$f$, 5$d$ and 6$s$ states in the valence one. Following this picture, the peak A in the O K edge is attributed to the mixing of the O 2$p$ states with the Gd 4$f$ ones and the broad peak B to their mixing with the more delocalized Gd 5$d$ and 6$s$ states. As illustrated by Figure IV.13, the intensity of peak A increases for small particles due to higher interaction of the Gd 4$f$ states with the O 2$p$ band. Moreover, it has been shown by XPS measurements that the binding energy of the Gd 4$f$ states is increased when Gd is oxidised which is in agreement with the increasing energy of the corresponding anti-bonding states (peak A)$^{[37]}$. This size induced energy shift of the Gd 4$f$ states can be directly correlated to the gap blue shift observed on Eu-doped Gd$_2$O$_3$ nanoparticles$^{[40]}$. For bigger particles (2.7 or 4 nm), the Gd 4$f$ electrons recover their strong atomic character, mixing with the O 2$p$ states is considerably reduced and the intensity of peak A breaks down. These experiments demonstrate the charge transfer from Gd to O in Gd$_2$O$_3$ and the deviation,
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at the nanoscale, from the stable trivalent configuration of Gd.

Modifications of the oxidation state of the rare earth in rare earth oxide nanoparticles were already reported for CeO$_{2-x}$ nanoparticles.$^{[41]}$ However, due to the stability of both the trivalent and quadrivalent form of Ce atoms, the reduction process implies the loss of one $f$ electron. Due to the strong stability of Gd in its trivalent form, such a mechanism is not possible in Gd$_2$O$_3$. We have demonstrated here that the charge transfer is rather led through a delocalization of a part of the $f$ electrons via hybridization with the valence band.

IV.1.2 Core-shell Gd2O3/SiOx

IV.1.2.1 Presentation of Core-shell Gd2O3/SiOx

Coating core Gd$_2$O$_3$:Tb$^{3+}$ with a shell of polysiloxane is a strategy which presents several advantages in the field of biological application. Apart ensuring core protection and water-solubility, the silica shell realizes two crucial functions. The first consists in allowing functionalization by biological groups since silica can be derivatized by organoalkoxysilanes containing reactive organic groups (e.g. amine, thiol, isothiocyanate). These organic functions which are covalently bound to the inorganic network act as anchorage sites for biomolecules. The second which was less extensively studied consists in increasing the optical response of luminescent cores used as optical tags. The enhancement of luminescence brought by coating can be explained on the basis of a reduction of the luminescence quenching of the surface hydroxyl groups. Another physical mechanism can also explain the modification of luminescence upon coating. It consists of an energy transfer between the two parts of the core-shell structure, the polysiloxane shell acting as an antenna which absorbs the light and transfers it to the core enhancing then its optical response.$^{[42]}$

A. Encapsulation of polysiloxane shell

In order to coat a polysiloxane shell, two precursors are chosen owing to the reactivity and compatibility with a further functionalisation:

- Tetraethylorthosilicate (TEOS; Si (OC$_2$H$_5$)$_4$) which is highly reactive and allows a good reticulation of the protective layer.

- Aminopropyltriethoxysilane (APTES;H$_2$N(CH$_2$)$_3$-Si(OC$_2$H$_5$)$_3$)

According to the chosen ratio (TEOS/APTES 40/60)
which provides the reactant groups (amines \(-\text{NH}_2\)) which can be used as grafting sites for the functionalization, in the polysiloxane layer.

Fluorophores are added in the polysiloxane layer by being mixed with APTES in dimethylsulfoxide (DMSO), which aims to produce fluorescence for biological detection.

Precursors need to be hydrolyzed in a solution of triethylamine TEA and water is necessary for a correct encapsulation. The different elements are added in sequentially, as well as precursors and hydrolysis solutions are added alternatively in the sample which is continuously stirred at 40°C. The process of encapsulation is shown in Figure IV.14.

Figure IV.14 Schematic representation of process of encapsulation of polysiloxane shell.

**B. Functionalization**

The functionalization is achieved by mixing encapsulated particles with diethylene triamine pentaacetic acid (DTPA) previously dissolved in DMSO. DTPA is a “chelating agent” that inhibits metal ions from combining with other ingredients in a product. DTPA has been used as a decontaminant for poison such as plutonium, etc. When it is involved in chelate complexes, the concerned ions are more readily eliminated. This aspect is very important as the elaborate particles should be eliminated rapidly from the organism, and they must not react with the organism and stay into their oxide form. It is important to notice that the functionalisation depends on the assigned application.
C. Purification

Purification of the obtained solution is necessary to prevent solvents from becoming useless and toxic. In this step of the experiment a dialysis against ethanol is usually performed. Currently the purification protocol is being improved by M. Garinot from Nano-H as following:

- Addition of diethyl ether (Et₂O)
- Centrifugation 3000rpm / 2’30”
- Dialysis against ethanol 95% (x2)
- Centrifugation 6000rpm / 4’30” between each dialysis
- The centrifugation pellet is taken up in water (filtrated 0.2 µm)
- Vivaspin 3000 MWCO: addition of water 8000 rpm / 15min (X 4: until the desired concentration)
- Conservation in freezer (-20°C) or room temperature or lyophilisation

IV.1.2.2 Optimization of Core-shell Gd₂O₃/SiOₓ

In this part, several kinds of polysiloxane shell with different thickness were coated to the same gadolinium oxide core by modifying the quantity of precursors added, which aims to follow up the growth of the polysiloxane shell according to the composition of the encapsulating solution.

IV.1.2.2.1 Encapsulation of different thickness of polysiloxane shell.

Gd₂O₃ cores doped with Tb³⁺ with a size of around 3.5 nm were prepared by the polyol method described in IV.1.1.2. The core solution at a concentration of 12 M was divided into four equal parts (25 ml for each part) for diverse encapsulations. For 1 mol Gd, precursor solutions (60%APTES and 40%TEOS) consisting of 2 mol, 4 mol, 6 mol and 8 mol SiOₓ were added and stirred for 48h at 40°C, and the corresponding obtained samples were denoted 2Si, 4Si, 6Si, and 8Si, respectively.

Figure IV.15(a) gives the evolution of the particle size obtained by PCS during the encapsulation. For all the samples, an increase of particle size with the time of encapsulation is observed. After reaction, the samples Gd₂O₃ coated with 4Si and 6Si per Gd present the same size evolution and the size reaches 5.5 nm at the end of encapsulation. The size of “sample 2Si” is about 4.8 nm and sample 8Si shows the largest size of 6.5 nm.

Figure IV.15(b) and (c) show the T₁ and T₂ evolutions in Nuclear Magnetic Resonance (NMR, see Appendix) experiments. There is no significant change of T₁ and T₂ for different encapsulating solution during the encapsulations. However, a visible leap occurred for both T₁ and T₂ as soon as encapsulating solution was introduced to core solution, which is a result of
the change of response of protons in magnetic field due to the change of solution composition. After the leap, the evolution of $T_1$ presents a slight increase, whereas that of $T_2$ shows a decrease before reaching the maximum value at the end of encapsulation.

Figure IV.15  (a) Particle size distribution (PSD); (b) $T_1$ (c) $T_2$ spectra of NMR experiment during encapsulation.
IV.1.2.2 Estimation of thickness of polysiloxane shell

The shell thickness of polysiloxane depends on the molar ratio of SiOx and Gd\textsuperscript{3+}, the size of core particle and the molar ratio of APTES and TEOS. In our work, the molar ratio of APTES and TEOS is fixed at 60/40, and therefore the shell thickness can be theoretically estimated on the base of core size (obtained from Volume PSD and changed from 1 to 5, systematically) and the molar ratio of SiOx and Gd\textsuperscript{3+} (changed from 2 to 8, systematically). The equations of calculation are as following:

\[
V_{Gd_2O_3} = \frac{4\pi}{3} \left( \frac{D_{Gd_2O_3}}{2} \right)^3
\]

\[
m_{Gd_2O_3} = V_{Gd_2O_3} \times d_{Gd_2O_3}
\]

\[
n_{Gd^{3+}} = \frac{2m_{Gd_2O_3}}{M_{Gd_2O_3}}
\]

\[
V_{SiOx} = \frac{n_{SiOx} \times M_{SiOx}}{d_{SiOx}}
\]

\[
D_{Gd_2O_3/ SiOx} = 2 \left[ \frac{3}{4\pi} \left( V_{Gd_2O_3} + V_{SiOx} \right) \right]^{1/3}
\]

\[
\alpha = \frac{n_{SiOx}}{n_{Gd^{3+}}}
\]

\[
e_{SiOx} = \frac{D_{Gd_2O_3/ SiOx} - D_{Gd_2O_3}}{2}
\]

\[
e_{SiOx} = \frac{D_{Gd_2O_3}}{2} \left[ \sqrt[3]{1 + 2\alpha \times \frac{d_{Gd_2O_3} \times M_{SiOx}}{M_{Gd_2O_3} \times d_{SiOx}}} - 1 \right]
\]

In these equations, the notations are the following: \( V \) = Volume, \( D \) = Diameter, \( m \) = Mass, \( d \) = volumic mass (\( d_{Gd_2O_3} = 7.407 \text{ g/cm}^3 \), \( d_{SiOx} = 2 \text{ g/cm}^3 \)), \( n \) = Molar number, \( M \) = Molar mass (\( M_{Gd_2O_3} = 362.5 \text{ g/mol} \), \( M_{SiOx} = 90.1 \text{ g/mol} \)), \( e \) = thickness, \( \alpha \) = molar ratio of SiOx/Gd.

The estimation results of the size of core-shell particles and the thickness of shell are shown in Table IV.1.
### Table IV.1 Thickness estimation of polysiloxane shell.

<table>
<thead>
<tr>
<th>$D_{Gd_2O_3}$ (nm)</th>
<th>Estimated $D_{Gd_2O_3/SiO_2}$ (nm)</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7</td>
<td>2.0</td>
<td>2.3</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>4.1</td>
<td>4.6</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>6.1</td>
<td>6.9</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.7</td>
<td>8.1</td>
<td>9.2</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8.4</td>
<td>10.1</td>
<td>11.5</td>
<td>12.5</td>
<td></td>
</tr>
</tbody>
</table>

### IV.1.2.2.3 Estimation of effective quantity of polysiloxane for encapsulation

The effective quantity of polysiloxane used for encapsulation was calculated and the results are displayed in Table IV.2.
Table IV.2 Effective quantity of polysiloxane.

<table>
<thead>
<tr>
<th>Samples</th>
<th>2Si</th>
<th>4Si</th>
<th>6Si</th>
<th>8Si</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{Gd_2O_3/SiO_x}$</td>
<td>4.26</td>
<td>5.61</td>
<td>5.97</td>
<td>6.75</td>
</tr>
<tr>
<td>Experimental shell thickness (nm)</td>
<td>0.455</td>
<td>1.13</td>
<td>1.31</td>
<td>1.7</td>
</tr>
<tr>
<td>Theoretical shell thickness (nm)</td>
<td>2.14</td>
<td>3.07</td>
<td>3.72</td>
<td>4.25</td>
</tr>
<tr>
<td>added quantity of polysiloxane (cm$^3$)</td>
<td>2.08E-20</td>
<td>7.28E-20</td>
<td>9.17E-20</td>
<td>1.41E-19</td>
</tr>
<tr>
<td>Effectively reacting quantity of polysiloxane (cm$^3$)</td>
<td>1.92E-22</td>
<td>6.73E-22</td>
<td>8.49E-22</td>
<td>1.31E-21</td>
</tr>
<tr>
<td>Effective molar ratio of Si/Gd</td>
<td>0.195</td>
<td>0.682</td>
<td>0.86</td>
<td>1.33</td>
</tr>
<tr>
<td>Effective reacting encapsulating solution (%)</td>
<td>9.75</td>
<td>17</td>
<td>14.3</td>
<td>16.6</td>
</tr>
</tbody>
</table>

From this table, we can find that only a small part (9.75%-16.6%) of encapsulating solution took part in the elaboration of polysiloxane shell. In the case of 2Si, the effective amount of encapsulating solution is the least.

IV.1.2.3 Visualization of Core-shell Gd$_2$O$_3$/SiO$_x$
IV.16 Double detection by shift of SPR peak and luminescence of nanoparticles-

Figure IV.16 TEM images of ‘2Si’ sample (a, b and c) and of ‘4Si’ sample (d) after encapsulation of APTES and TEOS.

The core-shell Gd$_2$O$_3$/SiO$_x$ particles were visualized by TEM. ‘2Si’ sample is shown in Figure IV.16 (a), (b) and (c). Particles size lie between 4-6nm which corresponds to the results obtained from PCS. Figure IV.16 (d) shows the TEM images of ‘4Si’ sample that presents a particle size of 6 nm approximately, which is also consistent with size obtained from PCS.

IV.2 A double biological detection system (SPR shift of Au dot arrays and luminescence of fluorophores encapsulated).

Metal thin films presenting a corrugation in the nanometer range exhibit a wide variety of optical properties that makes them the center of considerable attention in the field of subwavelength optics, light generation and bio-photonics. In this last domain, they can constitute the substrate of biological sensors liable to measure the concentration of a species (biomolecule, peptide, ssDNA…) after binding onto a specific ligand grafted on the substrate \[^{[43]}\]. The main principle for detection uses the localized surface plasmon resonance (LSPR) of the metal substrate. This latter which is very sensitive to any modification of the refractive index at metal surface undergoes a significant shift upon biomolecule binding. If a nanoparticle could be attached to the molecule, one could expect two advantages. The first advantage is related to the high hindrance of the particle which should induce a higher LSPR shift. This was already suggested in literature which reports the deposition of metal clusters \[^{[44]}\].
and CdTe quantum dots$^{[45]}$ (QD) on corrugated metal films. In this section, we will compare the kinetics of streptavidin adsorption on a biotinylated gold surface when streptavidin is free or attached to a particle with the aim to verify if its attachment to a particle effectively increases the detection sensibility. The second advantage is related to the stability upon heating of the inorganic particle. When deposing the solution containing streptavidin on the substrate, other molecules present in the solution can also be adsorbed and induce an unexpected LSPR shift. Then if all the organic molecules could be eliminated upon an optimized heating, only the fixation of streptavidin could be detected because it would be the only molecule attached to some inorganic matter. Two types of substrates are studied in this work: (i) Au dots arrays and (ii) flat or corrugated Au thin films. (i) Au particles arrays prepared by Electron Beam Lithography (EBL) are the substrates on which the kinetics of compared adsorptions is studied. It is justified because of the high efficiency of this type of substrate for the LSPR-based detection of streptavidin/biotin binding. (ii) Flat and corrugated films have a higher gold specific area: they then will be chosen for analyses or specific experiments which require a large quantity of matter like X-ray Photon Electron Spectroscopy (XPS). Corrugated films will be also chosen for assays requiring heating because they are prepared by Pulse Laser Deposition (PLD) at high temperatures (600°C) so that they do not undergo any LSPR shift for annealing at lower temperatures. If streptavidin is attached to a luminescent particle, the biotin/streptavidin binding can be also detected by the luminescence of the particle immobilized on the surface under appropriate excitation. This second type of detection again benefits of the fact that the object labeling the probe is a particle (and not for example a dye). Indeed, particles are generally brighter than single luminophore whatever they consist of quantum dots (QD) with high intensity emission$^{[46]}$ or form a platform incorporating multiple dyes$^{[47]}$. QDs are certainly good candidates and it is surprising that the two possibilities of detection (intrinsic luminescence, substrate LSPR shift) were examined only separately in their case. In this work, we propose an alternative and more complete approach already validated in a previous work$^{[48]}$ by using the doubly luminescent particles$^{[49]}$ that we developed for in-vivo imaging$^{[50]}$. They consist of a gadolinium oxide core encapsulated in a polysiloxane shell containing fluorescein allowing then three possibilities of detection (two intrinsic luminescence, substrate LSPR shift). We will show how the two types of luminescence (organic and inorganic) can be separately evidenced after particle fixation on the substrate opening then promising routes for multi-labeling strategies.

IV.2.1 Presentation of SPR

When a beam of light passes from material with a high refractive index (e.g. glass) into material with a low refractive index (e.g. water) some light is reflected from the interface. When the angle at which the light strikes the interface (the angle of incidence or $\theta$) is greater
than the critical angle ($\theta_c$), the light is completely reflected (total internal reflection). If the surface of the glass is coated with a thin film of a noble metal (e.g. gold), this reflection is not total; some of the light is 'lost' into the metallic film. There then exists a second angle greater than the critical angle at which this loss is greatest and at which the intensity of reflected light reaches a minimum or 'dip'. This angle is called the surface plasmon resonance angle ($\theta_{spr}$). It is a consequence of the oscillation of mobile electrons (or 'plasma') at the surface of the metal film. These oscillating plasma waves are called surface plasmons. The incoming light penetrates the interface into a less dense medium to a distance of one order of wavelength in the manner of evanescent waves or field.\[51\] The wave vector of the evanescent field ($K_{ev}$) is given by

$$K_{ev} = \frac{w_0}{c} \eta_g \sin \theta$$

Where $w_0$ is the frequency of incident light, $\eta_g$ the refractive index of the dense medium (glass), $\theta$ the angle of incidence of the light and $c$ the speed of light in a vacuum. The wave vector of a surface plasmon ($K_{sp}$) can be approximated to

$$K_{sp} = \frac{w_0}{c} \sqrt{\frac{\varepsilon_m \eta_g^2}{\varepsilon_m + \eta_g^2}}$$

Where $\varepsilon_m$ is the dielectric constant of the metal film and $\eta_g$ is the refractive index of the dielectric medium.\[52\] The evanescent wave of the incoming light is able to couple with the free oscillating electrons (plasmons) in the metal film when $K_{sp} = K_{ev}$, and thus the surface plasmon is resonantly excited, hence the term surface plasmon resonance.

The most common approach to excitation of surface plasmons is by means of a prism coupler and the attenuated total reflection method. There are two configurations of the attenuated total reflection method: Otto geometry and Kretschmann geometry. In the Otto setup (\textit{Figure IV.17}(a)), the light is shone on the wall of a glass block, typically a prism, and totally reflected. A thin metal (for example gold) film is positioned close enough, that the evanescent waves can interact with the plasma waves on the surface and excite the plasmons. In the Kretschmann configuration (\textit{Figure IV.17} (b)), the metal film is evaporated onto the glass block. The light is again illuminating from the glass, and an evanescent wave penetrates through the metal film. The plasmons are excited at the outer side of the film. The latter configuration is widely used within the designs of most SPR instruments.
IV.2.2 Deposition of Gd$_2$O$_3$/SiO$_x$ on Au substrate.

IV.2.2.1 Functionalization of core-shell Gd$_2$O$_3$@SiO$_x$ with streptavidin (SA)

The core-shell Gd$_2$O$_3$@SiO$_x$ particles were obtained with a mixed shell of tetraethylorthosilicate (TEOS), (3-aminopropyl) triethoxysilane (APTES) and rhodamine B isothiocyanate coupled to APTES (APTES-RBITC). These two compounds ensure the encapsulation of dyes (RBITC) within the shell and the presence of amino groups acting as anchoring sites at the outer surface.

For functionalization with streptavidin, a two-step strategy was chosen. First, a bifunctional spacer, the p-phenylene diisothiocyanate was grafted on particles by reaction between one of its isothiocyanate function and amino surface groups. The second isothiocyanate function which remains free is, in a second time, allowed to react with SA amino group. Through this process SA is then covalently linked to the particle. The size of the core particles and that of the core/shell ones were determined by Photon Correlation Spectroscopy (PCS) from the fluctuations of the scattered intensity under illumination with a HeNe laser (633 nm). The hydrodynamic oxide core was found equal to $1.4 \pm 0.1$ nm (mean standard deviation of $0.6 \pm 0.1$ nm) and that of the core/shell of $5.0 \pm 0.2$ nm (mean standard deviation of $1.6 \pm 0.1$ nm) indicating that the shell thickness is of $1.8 \pm 0.1$ nm with a mean standard deviation of $0.7$ nm. We demonstrated previously that the values deduced from PCS are in good agreement with those given by Transmission Electron Microscopy. The quantity of streptavidin was adjusted in order to obtain, under the assumption of a 100% yield, a functionalization ratio of one streptavidin per particle. The solution then contains $7.5 \times 10^{-6}$M of core/shell particles and $7.5 \times 10^{-6}$M of streptavidin.
IV.2.2.2 Binding of streptavidin labelled by core-shell particles on the substrates

As shown in the schematic system of multiple detection (Figure IV.18), to bind streptavidin labelled by core-shell particles on gold substrates, the latter were biotinylated by immersion during 2 hours in a solution (1 mg/ml) of tri-thiolated polypeptides modified with a biotin molecule at their N-term end and washed to remove all unbound molecules. The films were then immersed in an aqueous solution containing the streptavidin labelled by core/shell particles at a concentration of \([\text{SA}] = 7.5 \times 10^{-7}\) M.

IV.2.2.2.1 Experimental evidence of the binding of streptavidin labelled by core/shell particles on gold dot arrays prepared by EBL.
Figure IV.18 AFM images of sample 1 (lattice parameter 340 nm): (a), (b), Au dots and (c), (d), Au dots coated by luminescent nanoparticles.

Figure IV.19 which shows the AFM images of the dots arrays before and after functionalization indicates that an important modification of the dots topology was induced by streptavidin binding and not by a tip artifact. Indeed whatever the magnification, a net smoothing of the dots occurred during the process which could be consistent with a rather uniform deposition of labelling particles on gold. On the contrary, the morphology of flat silica remains essentially unchanged during the deposition indicating that no particles were adsorbed on it. The fixation of particles on the substrate is mainly caused by streptavidin/biotin coupling and is then highly specific.
**IV. Double detection by shift of SPR peak and luminescence of nanoparticles**

**Figure IV.20** Size distribution of sample 1: (a) Perimeter length and (b) height of Au dots, (c) perimeter length and (d) height of Au dots coated by luminescent particles.

**Table IV.3** Dimensions of Au dots before and after functionalization of luminescent nanoparticles (NP).

<table>
<thead>
<tr>
<th>Lattice parameter (nm)</th>
<th>Height (nm)</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Au</td>
<td>Au+NP</td>
</tr>
<tr>
<td>340</td>
<td>70 ± 2</td>
<td>77 ± 2</td>
</tr>
<tr>
<td>1140</td>
<td>71 ± 2</td>
<td>78 ± 2</td>
</tr>
</tbody>
</table>

**Table IV.3** and **Figure IV.20** give the height and the diameter of the dots deduced from AFM images. Diameter was obtained using the freeware image processing software WSxM\textsuperscript{[54]} from the distribution of the perimeter lengths of the recognized dots. The perimeter length of the dots is measured by the software from the part on the top of the dots. Calculated on low magnification images, the mean diameter is an average upon more than 300 dots. Height was obtained as an average upon more than 60 dots using height plots given by the software of the Digital Instruments Nanoscope Di3100 microscope. Whatever the distance between the dots
(340 or 1140 nm), their mean height and diameter undergo significant increases during functionalization whereas the related mean standard deviations of their distribution remain essentially unchanged. This is an additional proof that all the gold dots of the substrate are effectively coated by the core/shell particles. The height logically increases from $70 \pm 2$ nm to $77 \pm 2$ nm by a value of $7 \pm 4$ nm which is of the order of magnitude of the average size of the particle deposited ($5 \pm 0.2$ nm). At the same time, the diameter increases by approximately twice this value ($15 \pm 3$ nm). Again this indicates that the particles deposition is highly specific since in the case where particles should be indifferently deposited on gold and silica, the height (which is the difference between the position of the dot top and the flat surface) should not be modified. That the height or half-diameter increase ($7-8 \pm 1.5$ nm) does not exactly match the particle size (5 nm) can be easily understood by the fact that the particle is not in close contact with the gold dots but separated from them by a streptavidin molecule with a total length of around 6 nm. The height increase does not coincide either with the sum of the particle size and the biomolecules length. This implies that during AFM observations the particles should be slightly displaced under the tip. Such displacements are certainly due to the flexibility of the organic binding between surface and particles. They also indicate that, for the concentrations used during immersion of substrates in particles solution, the particles paving on gold dots is certainly not compact.

IV.2.2.2.2 Experimental evidence of the binding of streptavidin labelled by core/shell particles on flat films prepared by PLD.

The fixation of particles on the substrate is now directly visualized using AFM in the case of the gold films made by PLD by comparing their morphology and roughness before and after particles deposition (Figure IV.21 (a) and (b)). The duration of particles labeled streptavidin incubation with the substrate is 1 hour. For a better visualization, larger particles with a core size of 7 nm, a shell thickness of 4 nm and a total size of around 15 nm (mean standard deviation of 5.6 nm) were used. They are shown in the inset of Figure IV.2 (b). For corrugated films prepared by PLD at 600°C, no surface modification is visible by AFM upon particles deposition, the roughness before and after deposition having the same value of 1.7 nm. On the contrary for the flat films which are prepared at room temperature and have a roughness of 0.3 nm, this latter increases to 1.3 nm after deposition. This increase is correlated to the image of Figure IV.21 (b) which clearly evidences the presence of particles with the expected size (15 nm) on the substrate. The shape of the particles is not well defined which can be explained by some slight displacements of the particles under the tip due to the flexibility of their organic binding to surface. The paving density can be directly estimated from Figure IV.21 taken into account that ~ 110 particles with a diameter of 15 nm lye on a surface of 250 nm x 250 nm. It is equal to ~ 0.33. The
order of magnitude of the paving density is also confirmed by XPS experiments. Indeed, upon particles deposition the atomic ratio of gold decreases by a factor of 30%. Since on one hand the depth analyzed by XPS is of the order of 5 nm for both gold and core/shell particles and on the other hand the size of the core/shell particle is equal to about 15 nm, this decrease should correspond to the part of the gold film coated by the particles (i.e. the paving density obtained by XPS is 0.3). Moreover, the Gd/Si atomic ratio obtained by XPS is equal to 0.02±0.005 whereas the theoretical one (determined from the geometry of the core/shell structure) is of 0.14, in good agreement with that obtained by chemical analysis (0.15±0.1). XPS being mainly sensitive to the surface of the particle, this confirms that the particles deposited possess effectively a core/shell structure.

![AFM images of morphology and roughness (a) before and (b) after particles deposition](image)

**Figure IV.21** AFM images of morphology and roughness (a) before and (b) after particles deposition

IV.2.2.3 Realization of a double biological detection (LSPR shift and organic luminescence)

A. Detection by LSPR shift in the case of gold dots arrays

Visible extinction spectra were measured using a Jobin Yvon micro-Raman Spectrometer (Labram) in standard transmission geometry with unpolarized white light. The transmitted light is collected by an objective (×10; N.A = 0.25) from a zone of 30x30µm². All measurements were collected in air.
For a complete evaluation of the particles ability to evidence the biotin/streptavidin binding, the position evolution of the LSPR of the substrate was measured at all the different steps of the process. Before any chemical modification of the gold array with a parameter of 200 nm, this one is characterised by a LSPR wavelength at $\lambda_{\text{LSPR}} = 680$ nm.

**Figure IV.21** Smoothed extinction spectra: (1) Au dots before chemical modification, (2) Au dots after modification with biotin and (3) Au dots after modification with particles labelled SA ($[SA] = 7.5 \times 10^{-7} \text{M}$).
This is shown by the extinction spectrum of Figure IV.21. A slight red-shift of 3 nm is observed after the first chemical treatment aiming at depositing a covering biotin layer on gold. The LSPR wavelength is indeed shifted up to $\lambda_{\text{LSPR}} = 683$ nm. Such a shift which confirms the presence of the biotinylated fixing layer is in good rather agreement with that already measured in previous work. The next step consisted to bind particles labelled streptavidin to the gold dots so biotinylated. This was achieved by using a dilution of the luminescent nanoparticles solution to a concentration of $7.5 \times 10^{-7}$ M in particles and $7.5 \times 10^{-7}$ M in streptavidin. The LSPR wavelength was then measured and a $\lambda_{\text{LSPR}}$ value of 704 nm was found. Consequently the shift was of 24 nm compared to the starting Au nanoparticles (before chemical modification) (Figure IV.21). We should note here that the real shift due to the presence of the nanoparticles with streptavidin (SA) is of 21 nm only, because of the additional 3 nm shift corresponding to the presence of the biotin layer.

The shift of LSPR wavelength is due to a local change of refractive index or effective thickness of the layer (see equation 1):

$$\Delta \lambda = m \Delta n \left[1 - \exp \left(\frac{-2d}{l_d}\right)\right]$$

where $\Delta \lambda$ is the wavelength shift, $m$ is the sensitivity of our gold dots to the local refractive index, $\Delta n$ is the change in refractive index induced by the adsorbate ($\Delta n = n_{\text{adsorbate}} - n_{\text{air}}$), $d$ is the effective adsorbate layer thickness and $l_d$ is the characteristic evanescent electric field decay length. The sensitivity $m$ does not depend of the adsorbate and is equal to 200 nm per Refractive Index Unit (RIU) according to the calculations performed by the Van Duyne group. The value of $l_d$ which was calculated by the Finite Difference Time Domain (FDTD) method is equal to 27 nm. The index difference between air and the functionalized particles can be deduced from the indexes of the different parts of the particles ($n_{\text{core}}=1.88$, $n_{\text{shell}}=1.5$, $n_{\text{streptavidin}}=1.56$). Taking into account the fact that the core volume is small compared to that of the shell and that the size of the whole particle is comparable to that of streptavidin, a good approximation of the index difference is $\Delta n = 0.53$. The effective thickness of the particles deposited can then be deduced from the wavelength shift, $\Delta \lambda = 21$ nm. $d$ is equal to 3 nm. When comparing this value to the size of the particles (5 nm) and that of the streptavidin/biotin couple (6 nm), it can be concluded that the paving density is of 0.27. Compared to the maximal density obtained in the case of a hexagonal paving (0.90), the paving density of particles is 30% of the maximal one for the incubation concentration used here ($[\text{SA}] = 7.5 \times 10^{-7}$ M). This is consistent with AFM images which already indicated an incomplete paving of the particles deposited.
B. Detection by scanning near-field optical microscope (SNOM) images of the organic luminescence in the case of gold dots arrays.

To detect the presence of biomolecules through the collection of their luminescence, a scanning near-field optical microscope (SNOM) was used in a reflexion configuration (Figure IV.22). The SNOM is a commercial microscope (the TwinSNOM of Omicron Nanotechnology) and the optical images are obtained with the SCALA PRO Software. The SNOM tips were obtained by heating and pulling a monomode optical fiber with a commercial CO$_2$-laser based puller. The probes were then tapered and metallized with chromium and aluminium. Through this process, the resulting tips have an aperture of approximately 150 nm. They were then employed to illuminate the sample with an Argon laser at a wavelength of 514 nm corresponding to the excitation (absorption) wavelength of rhodamine ($\lambda_{\text{absorption}} \sim 514$ nm). Both the resulting scattered light and sample luminescence were collected by a Cassegrain collection objective (Figure IV.22). In order to measure exclusively the fluorescence of the rhodamine, a notch filter at 514 nm was intercalated before the detector to cut out the collected laser light. The signal obtained was then sent towards a photomultiplier tube connected to the SNOM.

![Figure IV.22](Image)

Figure IV.22 Simplified scheme of Scanning Near-field Optical Microscope.

After having demonstrated that the particles could be successfully used for detecting the streptavidin/biotin specific binding by measuring the LSPR shift that they induce on a real probed zone of 30×30µm$^2$, the next step is now to validate the SNOM technique as an additional route to detect the same interaction.

The principle of this detection technique consists to record optical images of gold dots arrays (luminescence) with the objective to detect a difference after fixation on the dots of the labelling nanoparticles. In a first step, we studied the substrate not yet functionalized and
characterized by a distance between the centers of two adjacent particles of 1140 nm. We obtained the uniform optical image given in Figure IV.23(a). No dot is distinguished because of a complete removing of the Rayleigh scattering on gold nanoparticles by the notch filter at 514 nm. In a second step, we recorded the same substrate functionalized by the particles encapsulating RBITC on a zone of ~ 3×3µm². We obtained the Figure IV.23(b) that we reproduced on various zones of the same substrate.

Figure IV.23 Optical images: (a) before and (b) after incubation of biotinylated Au dots arrays with streptavidin conjugated particles.

They clearly evidence that RBITC which is present on the substrate is strongly localized on the gold dots. Indeed, the spots form a lattice quite similar to that of the dots (p4mm with a lattice parameter equal to 1.14µm). The size of the spot (200 nm) is also in good agreement with the size of the coated dots at their basis (200 nm) a value slightly larger than that given in Table 1 (160 nm) corresponding to the diameter at middle height. These results confirm the high selectivity of the labelling particles already inferred from AFM observations.

In summary, our results indicate that the gold arrays elaborated by EBL used in combination with particles encapsulating dyes are good candidates for increasing the reliability of biological detection. Indeed they constitute the bases for new devices permitting two types of detection techniques. The streptavidin/ biotin binding was indeed successfully evidenced with a shift of the localized surface plasmon resonance of the gold dots and with the luminescence of the fluorophores encapsulated.

IV.2.2.4 Detection by LSPR shift upon heating.

In this part we aim at performing annealing in order to remove all organic molecules. Since the substrates are annealed at a temperature of 250°C for 1 hour in order to eliminate all the organic molecules, the rough films prepared by PLD at high temperature
(600°C) are the ones which are chosen in this part.

Figure IV.24  (a) and (b) Extinction spectra of gold films elaborated at 600°C as prepared (○ or ◇), biotinylated (△), biotinylated and grafted with particles labelled streptavidin (□) before annealing at 250°C under air. Extinction spectra of the same films as prepared (○), biotinylated (▲), biotinylated and grafted with particles labelled streptavidin (■) after annealing at 250°C under air.

The evaluation of the streptavidin/biotin binding by the LSPR shift of the substrate is again measured at all the different steps of the process. Before biotinylation, the substrate is characterized by a LSPR wavelength at $\lambda_{LSPR} = 617$ nm (Figure IV.24(a)). Figure IV.24 (a) shows also that a slight red-shift of 5 nm is observed after the first chemical treatment aiming at deposing a biotin layer on gold. The LSPR wavelength is indeed shifted up to $\lambda_{LSPR} = 622$ nm. The next step consisted to bind particles labeled streptavidin to the gold film so
Double detection by shift of SPR peak and luminescence of nanoparticles

Biotinylated. This was achieved by incubating the substrate with a dilution of the luminescent particles solution to a concentration of $7.5 \times 10^{-7}$ M in particles and $7.5 \times 10^{-7}$ M in streptavidin for 1 hour. The LSPR was then measured and $\lambda_{\text{LSPR}} = 640$ nm was found. The real shift due to the presence of the nanoparticles with streptavidin is then of 18 nm.

The size of the particles is 5 nm and that of the streptavidin/biotin couple of around 6 nm. Then if the film was uniformly coated by a dense layer of particles, the shift observed would be equal to $\Delta \lambda_{\text{max}} = 48$ nm. In a very simple model where only the part of the surface coated by the particles contributes to the shift, this latter is related to the particles surface density, $p$, according to the relation: $\Delta \lambda = p \Delta \lambda_{\text{max}}$. $\Delta \lambda$ being equal to 18 nm, it can be concluded that the paving density, $p$, is around $0.38 \pm 0.04$. Compared to the maximal density obtained in the case of a hexagonal paving (0.90), the compacity of the particles layer is of about 42% for the incubation duration and concentration used (1 hour, [SA] = $7.5 \times 10^{-7}$ M). This is consistent with AFM images and XPS results which already indicated a paving of this order of magnitude ($0.33 \pm 0.06$ and $0.3 \pm 0.06$ respectively).

After annealing of the rough gold film at 250°C for 1 hour, the extinction spectrum does not change as one can see in Figure IV.24 (a) which shows a perfect coincidence between the spectra before (○) and after annealing (●). For better clarity, the two spectra are indexed by the same symbol (○). This result is then the confirmation that the rough film which was elaborated at 600°C does not undergo any structural modification during further annealing at lower temperature. Concerning the biotinylated film, Figure IV.24 (b) shows that the annealing at 250°C blue-shifts the LSPR wavelength from 622 nm to its value before biotinylation (617 nm). This is the proof that during annealing all the biological species deposed on the film are totally removed. On the contrary, the biotinylated films incubated with particles labelled streptavidin only undergo a blue LSPR shift of 3 nm with annealing. These annealed films then present a LSPR shift of 20 nm relatively to the (annealed) gold film so that, as expected, the detection of the biotin/streptavidin binding is still possible after annealing. This value of 20 nm is in good agreement with the theoretical shift (18 nm) calculated for particles paving with a density of 0.38 the gold rough films studied.

IV.2.2.5 Double optical detection (organic and inorganic luminescence)

The possibility of detecting the organic luminescence of the dyes encapsulated in the shell of the particles was already demonstrated in the Figure IV.23 (a) and (b). Before annealing, this high luminescence completely masks that of the terbium doped core. However, one could expect that the annealing at 250°C, by degrading the luminescence of the dyes, should reveal that of terbium. Figure IV.25 shows that it is not the case. Two reasons can
explain this absence of luminescence. The first one is that like many other cations such as Fe, V, Mo \cite{60}… Tb can exist under different valences (Tb$^{3+}$ and Tb$^{4+}$). Then during annealing under air, Tb$^{3+}$ oxidizes to Tb$^{4+}$, a cation which is not luminescent. The other one is that, the crystallinity of Gd$_2$O$_3$:Tb obtained by the polyol method is very low\cite{61}. To both increase the crystallinity and preserve the valence of the terbium cations, an annealing at 450°C under a hydrogen/argon mixture containing 5\%vol of H$_2$ was performed. The resulting luminescence is shown in Figure IV.25. Two peaks at 488 and 545 nm corresponding to the $^5$D$_4 \rightarrow ^7$F$_5$ and $^5$D$_4 \rightarrow ^7$F$_6$ transitions clearly emerge from the noise. As already shown for reducing annealing at this temperature, these peaks are structured, each transition being splitted into several fine peaks corresponding to the level of degeneracy of the transition\cite{62}.

![Figure IV.25 Tb luminescence of the gold films biotinylated and incubated with particles labelled streptavidin after annealing under air for 1 hour (solid line) or under an hydrogen/argon mixture for 1 hour (bold line).](image)

The proof of concept that the inorganic luminescence can be detected from a monolayer of particles with a small core of 1.4 nm opens interesting strategies for multiple labelling. Taken into account that particles are always detected by LSPR, different labels can be constituted by crossing organic and inorganic luminescence. We demonstrated previously \cite{48} that the particles could encapsulate rhodamine B isothiocyanate (RBITC) (Figure IV.28(a)). Then, three different shells have already been elaborated: that containing fluorescein isothiocyanate (FITC) (Figure IV.28(b)), that containing RBITC and that containing no dyes. Here two different cores have been tested: that containing Tb$^{3+}$ and that containing no luminescent lanthanide. Therefore we presently
have six different labels. The number of labelling particles can be enormously increased by
crossing different dyes (Cyanins, Alexa…) and different lanthanides from Eu (luminescing in
the visible) to Nd (luminescing in the infra-red). Moreover, contrary to what happens at a
macroscopic scale, energy transfer between lanthanides does not occur on the nanometre scale
[62]. The possibilities of different emission from lanthanides combinations in a same core are
then quite infinite.

IV.2.2.6 The effect of labelling streptavidin by a particle upon binding kinetics.

To evidence the fact that attaching a particle to the streptavidin modify the kinetics of its
binding to biotin, the LSPR shift $\Delta \lambda$ was measured for different incubation times between the
biotinylated gold dots arrays and on one hand free streptavidin (dash line in Figure IV.26 (a))
and on the other hand particle labelled streptavidin (solid line in Figure IV.26 (a)). The
kinetics was also followed by the luminescence of the FITC encapsulated in the particles
bound to the substrate (Figure IV.23 (a) and (b)). Three observations can be made. First
streptavidin binding to the substrate starts more rapidly when it is attached to a particle (10
minutes after the beginning of incubation) rather than when it is free (16 minutes). Second,
the LSPR shift reaches more rapidly its final value when streptavidin is labelled by particles.
For example for an incubation duration of 1 hour, the LSPR shift only attains $\sim50\%$ of the
final shift when streptavidin is free instead of 75% when it is attached to a particle. This
kinetics enhancement could be explained by the greater inertia of the streptavidin labelled (the
presence of the particle increases the mass of streptavidin of 120%). The third observation
concerns the final LSPR shift attained after saturation. It is greater when streptavidin is
labelled by particles ($\Delta \lambda = 33 \text{ nm}$) than when it is free ($\Delta \lambda = 28 \text{ nm}$) which indicates that not
only the rapidity but also the sensitivity of the assay can be increased by particles labelling.
To go further in the interpretation, one can calculate the number of streptavidin molecules
bound to the biotinylated dots in each case. When streptavidin is used alone, it would induce a maximal shift, $\Delta \lambda_{\text{max}}$, of 40 nm if it formed a dense layer of protein on gold dots. The streptavidin paving density at saturation is then equal to 0.70 (Figure IV.26 (b)). When streptavidin is labelled by particles, it would induce a $\Delta \lambda_{\text{max}}$ of 59 nm so that, at saturation, the paving density of particles labelled streptavidin is only 0.55. Figure IV.26 (b) details the whole kinetics of streptavidin binding by plotting the evolution of the paving (relative) density of streptavidin on dots surface in the two cases. The results mean that when streptavidin is ballasted by a particle, less proteins can, at saturation, bind to gold surface. However, even if the final protein density is smaller when a particle is attached to the protein (0.55 instead of 0.70) the effect upon the LSPR shift is greater (33 nm instead of 28 nm). Attaching a particle to streptavidin is then beneficial for both accelerating the binding kinetics and increasing the detection sensitivity.

We can compare the paving density of particles labelled streptavidin when it is bound to dots arrays or rough films. For an incubation duration of 1 hour, its paving density is equal to 0.42±0.04 when it is bound to dots arrays and to 0.38±0.04 for rough films (Figure IV.26 (b)). An important result is then that regardless of the type of the biotinylated substrate (gold dots arrays prepared by EBL or rough gold films elaborated by PLD), the grafting efficiency of particles labelled streptavidin is the same. The difference in the LSPR shift observed (25 nm for the dots arrays, 18 nm for the rough films) is uniquely due to a difference in the parameters $m$ and $l_d$ of the two types of substrates i.e. to their sensitivity.
IV.3 Conclusions

Nanostructured Tb$^{3+}$-doped Gd$_2$O$_3$ particles have been synthesized from chloride precursors by NaOH addition in a polyol medium. *In-situ* luminescent spectra have been investigated in order to follow up the process of formation and growth of these particles by varying parameters as the elaboration temperature and the rate of NaOH addition. Contrarily to all the literature related to the “polyol” synthesis, particles can be directly formed at room temperature. These particles are also slightly bigger and organized in nanorods when NaOH is added progressively. It is found that the influence of annealing up to 160°C strongly depends on the NaOH addition rate. Annealing leads to bigger particles only in the case of a progressive addition of NaOH.

The size induced modifications of the electronic structure of the nanoparticles Gd$_2$O$_3$ in different sizes were investigated by EELS. In the oxygen K spectra, the observed pre-edge peak A is attributed to the mixing of the O $p$ states with Gd 4$f$ ones and the broad peak B (540eV) to their mixing with the more delocalized Gd 5$d$ and 6$s$ states. The intensity of peak A increases for small particles (1.1 and 1.5nm) due higher interaction of the Gd 4$f$ states with the O 2$p$ band, which is consistent with the results obtained in spectra of Gd N$_{4,5}$. The experiments demonstrate the charge transfer from Gd to O in Gd$_2$O$_3$ and the deviation from the stable trivalent configuration of Gd.

The gold arrays elaborated by EBL used in combination with particles encapsulating dyes are good candidates for increasing the reliability of biological detection. Indeed they
constitute the bases for new devices permitting two types of detection techniques. The streptavidin/biotin binding was indeed successfully evidenced with a shift of the localized surface plasmon resonance of the gold dots and with the luminescence of the fluorophores encapsulated.
Rerefences:


-IV.Double detection by shift of SPR peak and luminescence of nanoparticles-


-IV. Double detection by shift of SPR peak and luminescence of nanoparticles-


V. Surface-enhanced chemiluminescence by rough metal films
In this chapter, we are interested in another detection system based on surface-enhanced chemiluminescence (SECL) brought by rough metal films. The phenomenon of SECL was demonstrated on the corrugated gold film supporting a catalyst (the peroxidase) of the chemiluminescence of luminol. Several parameters were investigated such as the distance between the film and the peroxidase, the nanostructure of thin films, the pH value of the luminol solution and the chemical nature of the thin films. Finally, the mechanism of SECL was discussed.

V.1 Surface enhanced chemiluminescence (SECL) on the gold thin films

V.1.1 Presentation of chemiluminescence (CL)

Chemiluminescence is the generation of electromagnetic radiation as light by the release of energy from a chemical reaction. It can be grouped into two types: direct luminescence and indirect luminescence. Most simply, given reactants A and B, product C can be obtained. The decay of the excited state C to a lower energy level is responsible for the emission of light. Such a process is called direct luminescence. If C (the donor) transfers its energy to F (the acceptor) and induces the decay of the excited state F to a lower energy level, this will result in some light emission. This process is called indirect luminescence.\textsuperscript{[1,2,3]}

In order that, a chemical reaction can produce chemiluminescence, the following conditions must be met: firstly, the chemical reaction must provide sufficient excitation energy; secondly, the energy produced by the chemical reaction can be accepted by the acceptor and then the acceptor is excited; thirdly, the molecules in excited state must have adequate quantum efficiency to release photons, or transfer energy to another molecule and make it being excited to release photon. Figure V.1 shows the CL spectra of reaction of luminol and H$_2$O$_2$ with an emission peak about 450nm wavelength. The HorseRadish Peroxidase is often added as a catalyst. Nowadays this has been widely used for biological detection, but the knowledge of the exact reaction
**Figure V.1 CL spectra of luminols**

mechanism is still incomplete. The chemiluminescent reaction can be roughly divided into two steps as displayed in **Figure V.2**: at first luminol in excited state in the presence of peroxide as a catalyst is turned into Precursor Aminophthalate*; then Aminophthalate* undergoes subsequent decay to the ground state (Aminophthalate) through the emission of a photon.\[4,5,6,7]\n
---

**Figure V.2 Chemiluminescent reaction process of luminols**
V.1.2 Demonstration of SECL

Gold films prepared by PLD technique from 30 to 700°C were used to study SECL. As shown in Table V.1, flat and smooth films are obtained when the substrate is maintained at room temperature whereas corrugated surfaces are obtained for temperatures higher than 200°C. According to Figure V.3, the flat films exhibit the mean features of the extinction of gold bulk \cite{8}, whereas for heating at temperatures higher than 200°C the films present a pronounced SPR extinction peak characteristic of the presence of clusters \cite{9}. This peak is located at about 600 nm and presents a blue shift with increasing the temperature of the substrate (Table V.1).

![Figure V.3 Absorption spectrum of gold films for different substrate temperatures: 30°C, 200°C, 300°C, 500°C, and 700°C, respectively.](image)

\[\text{Absorption (a.u.)} \]

\[\text{Wavelength (nm)} \]

\[\text{425 nm} \]

\[\text{200°C} \]

\[\text{300°C} \]

\[\text{700°C} \]

\[\text{500°C} \]

\[\text{Room temperature} \]
Table V.1  Characteristics of the samples elaborated by PLD.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>SPR position (nm)</th>
<th>SPR intensity</th>
<th>Roughness (nm)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>No peak</td>
<td>No peak</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>200</td>
<td>670</td>
<td>0.23</td>
<td>1.3</td>
<td>~15</td>
</tr>
<tr>
<td>300</td>
<td>635</td>
<td>0.21</td>
<td>1.5</td>
<td>~20</td>
</tr>
<tr>
<td>400</td>
<td>625</td>
<td>0.37</td>
<td>1.5</td>
<td>~25</td>
</tr>
<tr>
<td>500</td>
<td>625</td>
<td>0.40</td>
<td>1.6</td>
<td>~30</td>
</tr>
<tr>
<td>600</td>
<td>620</td>
<td>0.42</td>
<td>1.9</td>
<td>~35</td>
</tr>
<tr>
<td>700</td>
<td>610</td>
<td>0.43</td>
<td>2.0</td>
<td>~50</td>
</tr>
</tbody>
</table>

After elaboration of the substrates, luminol CL can be generated at their vicinity following a process described in Figure V.4. Firstly, tri-thiolated polypeptides containing 11 amino-acid and modified with a biotin molecule at their N-term end were spotted as 1.2 nl drops (1 mg/ml) on the Au thin films through an automatic piezoelectric spotter (BCA1, Perkin Elmer). The peptides were incubated during 2 hours and then washed to remove all unbound molecules. In a second step, the treated films were immersed for 20 minutes in veronal buffer solution (VBS) containing additional 1% bovin serum albumin (BSA) and 0.1% polyoxyethylenesorbitan monolaurate (Tween) and finally incubated with peroxidase labelled streptavidin (1 µg/ml) for 30 minutes. The CL measurements were taken with a –30°C cooled CCD camera (Intelligent Dark Box II, Fuji Film). After immobilization of labelled streptavidin, the samples were dipped into a VBS solution containing luminol and additives agents favoring its CL (220 µM luminol, 500 µM H2O2 and 200 µM p-iodophenol). The light emitted by the luminol brought at peroxidase vicinity was integrated for 10 sec. The images obtained were quantified and the results were given in arbitrary units (a. u.) and each value was an average of four measurements.
Figure V.4 Schematic processes of SECL experiments.

Figure V.5 Chemiluminescence intensity induced by peroxidase labeled molecules physisorbed 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.

Figure V.5 which displays the CL intensity near Au thin films clearly demonstrates that a net enhancement is induced by films corrugation. Indeed, the luminescence is considerably stronger for the films which present the optical properties characteristic of clustered samples (those made at substrate temperatures higher than 200°C). For instance, the intensity of the...
luminescence emitted by luminol is around 1700 at vicinity of the flat bulk like film prepared at 30°C, whereas it is comprised between 17 000 and 26 000 near the corrugated films prepared above 200 °C. Inset in Figure V.5 also illustrates this phenomenon: the images of the CL spots are more clearly visible when the substrate is corrugated (preparation at 700°C) than when it is flat (preparation at RT=30°C).

V.2 Optimization of SECL
V.2.1 Influence of the distance between substrate and peroxidase

A few influencing factors were studied to understand the mechanism of SECL, among them the distance to metal substrate, the substrate morphology and the pH. To evaluate the effect of the distance between the emitting luminol and the substrate, different peptides differing from their chain length were used. As displayed in Figure V.6, peptides containing 5, 11, 17, or 23 amino acids (denoted respectively as P5, P11, P17 and P23) and having a length, d, of about 1.3, 3.5, 5.7 and 7.8 nm were spotted to get different separation distances between substrate and peroxidase (Table V.2). Besides the bulk-like and cluster-like Au films, bulk-like and cluster-like Au-Ag films were prepared to be compared with Au films. Characteristics of the films are displayed in Table V.3. Roughness is defined using atomic force microscopy (AFM) as the standard deviation of the height value within a box cursor with a size of 250x250 nm² and a resolution of 256x256.

Table V.2 Characteristics of peptide and the distance between peroxidase and metal.

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>Length of peptide (nm)</th>
<th>Length of peptide +SAV (nm)</th>
<th>Distance between peroxidase and metal (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5</td>
<td>C_{33}H_{57}N_{9}O_{9}S_{4}</td>
<td>1.34</td>
<td>7.34</td>
</tr>
<tr>
<td>P11</td>
<td>C_{65}H_{99}N_{21}O_{27}S_{4}</td>
<td>3.5</td>
<td>9.5</td>
</tr>
<tr>
<td>P17</td>
<td>C_{81}H_{123}N_{29}O_{33}S_{4}</td>
<td>5.66</td>
<td>11.66</td>
</tr>
<tr>
<td>P23</td>
<td>C_{105}H_{153}N_{39}O_{45}S_{4}</td>
<td>7.82</td>
<td>13.82</td>
</tr>
</tbody>
</table>

SAV: streptavidin.
Figure V.6  Chemical structures of biotin and different chain length peptides.
Table V.3 Characteristics of the samples studied.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substrate temperature during deposition</th>
<th>SPR Peak position (nm)</th>
<th>SPR HWHM (nm)</th>
<th>Roughness (nm)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat Au</td>
<td>RT</td>
<td>No peak</td>
<td>No peak</td>
<td>0.3</td>
<td>No particle</td>
</tr>
<tr>
<td>Flat Au-Ag</td>
<td>RT</td>
<td>No peak</td>
<td>No peak</td>
<td>0.4</td>
<td>No particle</td>
</tr>
<tr>
<td>Rough Au</td>
<td>600°C</td>
<td>690</td>
<td>110</td>
<td>1.9</td>
<td>30</td>
</tr>
<tr>
<td>Rough Au-Ag</td>
<td>200°C</td>
<td>690</td>
<td>190</td>
<td>4.1</td>
<td>30</td>
</tr>
</tbody>
</table>

\[ \eta_q = \frac{1}{\left(\frac{3}{4}\right)^4 + 1} \]

**Figure V.7** Luminol-H\(_2\)O\(_2\) chemiluminescence induced by peroxidase on (flat) bulk-like Au (circles) and Au–Ag films (squares) with different metal/peroxidase distances. The lines are fits of these data according to a Förster process simulation. Insert: absorption spectra of the flat films.

**Figure V.7** shows the results of luminol CL near bulk-like Au and AuAg films respectively. In both cases, the CL intensity increases with the distance and reaches about 2,000 in arbitrary units (a.u.). Assuming that in absence of clusters, there is no catalytic effect, this increase should be entirely related to the decrease in metal quenching with the distance. Förster transfer theory predicts that such a quenching possesses an inverse fourth power dependence with the distance \(d^{d-4}\) between donor and acceptor:
\[ \eta_q \approx \left(1 + \left(\frac{d_0^{d_{-a}}}{d^{d_{-a}}}ight)^4\right)^{-1} \] [10]. In this relation \( d_0^{d_{-a}} \) is a critical distance between donor and acceptor and \( \eta_q = \frac{I}{I_0} \) is the ratio of dye fluorescence in presence of a film at the distance \( d^{d_{-a}} \), \( I \), and that in absence of the film, \( I_0 \). Figure V.7 shows that in the case of both Au and Au-Ag flat bulk-like films, such a relation is satisfied between the fluorescence intensity \( I \) and the distance \( d \) between the film and the catalyzing peroxidase. A same fit \[ \eta_q = \frac{1}{\left(\frac{3}{d}\right)^4 + 1} \] is effectively obtained for the experimental data relative to both films with \( d \) expressed in nanometers. The critical value found, \( d_0^{\text{quench}} = 3 \pm 0.5 \text{ nm} \) for both Au and Au-Ag films indicates then that the critical distance \( d_0^{d_{-a}} \) between the film and the luminol is in good agreement with the order of magnitude given by Förster for the critical donor/acceptor distance, between 5 and 10 nm. For distances \( d \) larger than 6 nm, the fluorescence reaches a plateau at a value which is independent of the chemical nature of the film (1800 for Au and 2000 for Au-Ag).
Figure V.8 (a) Luminol-H$_2$O$_2$ chemiluminescence induced by peroxidase on cluster-like Au (circles) and Au–Ag films (squares) with different metal/peroxidase distances. Insert: absorption spectra of the rough films. (b) Enhancement factor: experimental data (symbols) and data fit (line). The dash lines are guides for the eye.

Completely different is the CL signal near cluster-like Au or Ag films. Indeed, Figure V.7 shows that the CL intensity first strongly increases with the distance between 1.3 nm to 3.5 nm and then slowly decreases after 3.5 nm. For a peroxidase/metal distance of 3.5 nm (P11) the intensity reaches high values around 30,000 in the same arbitrary units. As evidenced previously, this increase of more than one order of magnitude compared to the CL intensity near flat films (Figure V.7) must be attributed to an enhancement of the fluorescence by the presence of clusters. To provide a quantitative information upon this enhancement, a simple model in which the intensity observed is the product of the quenching factor $\eta_q (\leq 1)$ and an enhancement one $\eta_e (\geq 1)$ can be proposed: $I = \eta_q \eta_e I_0$. The enhancement factor $\eta_e$ (Figure V.8(b)) can thus be derived from the relation: $\eta_e = I/I_0$, where $I$ is given in Figure V.8(a) and $I_0$ in Figure V.7.

As expected, the $\eta_e$ evolution is found to decrease with the distance. However, the decreasing trend is smoother than that relative to quenching. Indeed, when fitting $\eta_e$ according to a relation similar to that relative to $\eta_q$: $\eta_e = 1 + \left(\frac{d_0}{d}\right)^n$ where $n$ is an integer and...
$d_0^{\text{enhanc.}}$ is a critical distance for enhancement, one finds that $n$ is much smaller than $4$ the exponent related to quenching and that $d_0^{\text{enhanc.}}$ is much larger than $d_0^{\text{quench.}} \approx 3$ nm. Precisely,

$$\eta_e = 1 + \left( \frac{220}{d} \right)^{0.7} \text{ for Au and } \eta_e = 1 + \left( \frac{350}{d} \right)^{0.7} \text{ for AuAg with } d \text{ expressed in nm.}$$

The relative variations of quenching and enhancement explain the particular behavior of CL intensity with distance observed in Figure V.8(a). At short distance, quenching predominates so that CL is low, whereas when increasing the distance, the enhancing mechanism dominates. That explains the presence of a maximum at a distance of 3.5 nm (for P11) slightly larger than $d_0^{\text{quench.}}$.

### V.2.2 Influence of the morphology of gold film

To evaluate the influence of the corrugation of the films upon SECL, different morphologies summarized in Table V.4 were investigated: flat films elaborated by PLD at RT, films with random roughness elaborated by PLD at 600°C and films with a controlled roughness elaborated by NSL using single and double layers masks. In all cases, a polypeptide with a chain length of 7.8 nm (P23) was used to separate the peroxidase from the Au surface.
Table V.4 Characteristics of the films elaborated.

<table>
<thead>
<tr>
<th>Samples</th>
<th>In-plane particle size (nm)</th>
<th>Out-of-plane particle height (nm)</th>
<th>Curvature radii (nm)</th>
<th>SPR peak (nm)</th>
<th>SPR intensity</th>
<th>CL intensity (a.u.)</th>
<th>Au surface ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoparticle Arrays</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL Au17</td>
<td>50</td>
<td>17</td>
<td>25</td>
<td>565-802</td>
<td>0.08</td>
<td>17800</td>
<td>25.5</td>
</tr>
<tr>
<td>SL Au24</td>
<td>50</td>
<td>24</td>
<td>25</td>
<td>583-715</td>
<td>0.09</td>
<td>20300</td>
<td>32.7</td>
</tr>
<tr>
<td>SL Au33</td>
<td>50</td>
<td>33</td>
<td>25</td>
<td>620-722</td>
<td>0.10</td>
<td>19700</td>
<td>42</td>
</tr>
<tr>
<td>DL Au18</td>
<td>30</td>
<td>18</td>
<td>15</td>
<td>667</td>
<td>0.05</td>
<td>8860</td>
<td>8.8</td>
</tr>
<tr>
<td>Flat Au film</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Au RT</td>
<td>/</td>
<td>25</td>
<td>/</td>
<td>No peak</td>
<td>/</td>
<td>1800</td>
<td>100</td>
</tr>
<tr>
<td>Rough Au film</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Au 600</td>
<td>35</td>
<td>25</td>
<td>2-3</td>
<td>620</td>
<td>0.23</td>
<td>20300</td>
<td>120</td>
</tr>
</tbody>
</table>

‘Au surface ratio’ denotes the gold area normalized to that of supporting glass. “SL” and “DL” denote single layer and double masks, respectively.

The CL intensity of luminol at the vicinity of peroxidase on the glass, flat and corrugated Au films and particle arrays are shown in Figure V.9. Much stronger luminol CL is observed with metal films presenting some corrugation (random or periodic). It is consistent with the already obtained results 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.\[^{11,12,13}\] 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.\[^{14,15,16}\] That explains why the CL measured is low at small metal/peroxidase distance (P5) and more important at larger one (P23) for which quenching is found to be completely negligible.\[^{17}\] 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 20000 and 18000 in the case of the larger one (P23).

**Figure V.9** Chemiluminescence intensity of luminol at the vicinity of peroxidase on the glass, flat Au film prepared at room temperature (AuRT), corrugated Au film prepared at 600°C (Au600), nanoparticle arrays fabricated by single layer mask (SLAu) and double masks (DLAu) with different particle heights (17 nm, 24 nm, 33 nm, 18 nm), respectively.

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). **Figure V.10** shows that the area-normalized luminescence is of almost one order of magnitude higher in the case of the particles arrays than for randomly corrugated films which is an indication that the roughness morphology is a key parameter for CL enhancement.
Surface-enhanced chemiluminescence by rough metal films

Figure V.10 Chemiluminescence intensity per unit area of Au on the flat Au film prepared at room temperature (AuRT), corrugated Au film prepared at 600°C (Au600), nanoparticle arrays fabricated by single layer mask (SLAu) and double masks (DLAu) with different particle heights (17 nm, 24 nm, 33 nm, 18 nm), respectively.

A particle-mediated transfer in which the electrons at vicinity of highly curved surface are the ones which cause some electron transfer from gold clusters to adsorbed H$_2$O$_2$ and permits to produce the key intermediate hydroxy and hydroperoxide radicals has already been proposed to explain the enhancement of CL in the case of particles [18]. The authors showed that the transfer was favoured for particles sizes comprised between 20 and 40 nm. 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 CL enhancement. On the contrary, the dots top of the particles arrays dots are characterized by curvature radii that are approximately half of the particles in-plane diameter given in Table 3 (25 nm for SL arrays, 15 nm for DL arrays) and then lie in the ideal range inducing the maximal enhancement (15 nm).

V.2.3 Influence of the pH value

Among the factors influencing the reactive conditions between luminol and hydrogen peroxide, pH, which is involved in the light emitting steps of luminol oxidation, was found to
be one of the more important \cite{19}. In order to study the influence of pH value, a biotinylated peptide with a length sufficiently small, 3.5 nm, (P11) to ensure a significant catalytic effect without correlative light emission quenching by metal, In the process of CL measurement, the films were divided in eight compartments separated by a hydrophobic layer and the pH of each compartment was adjusted to values varying from 7 to 12 (7, 8, 8.5, 9, 10, 11, 11.5, 12) by addition of suitable quantities of NaOH (5N) or HCl (6N).

The reaction of luminol/H$_2$O$_2$ catalyzed by peroxidase involves two principal stages: (i) the first one is the enzymatic stage appearing as the sequence of the following reactions (R) \cite{20}:

\[
P + H_2O_2 \rightarrow PI + H_2O
\]

\[
PI + L \rightarrow PII + L^-
\]

\[
PII + L \rightarrow P + L^- + H_2O
\]

where P, PI, and PII are peroxidase and two intermediate complexes respectively; L and L$^-$ are luminol and the product of free-radical single-electron oxidation respectively. (ii) The second stage involves the transformation from L$^-$ to 3-aminophthalate in an excited state which results in light emission \cite{21}. In presence of peroxidase \cite{22}, luminol CL is maximal for pH ranging between 8 and 9 whereas in absence of this catalyst \cite{23,24}, the maximum of CL locates at 10-11 pH value.

The influence of pH upon luminol/hydrogen peroxide CL was first investigated for reactions occurring near flat surfaces. \textbf{Figure V.II} which displays the CL intensity as a function of pH on flat glass, Au and Ag films clearly shows two different types of behaviour for, on one hand, the glass support and, on the other hand, the metal films. For glass (\textbf{Figure V.II}(b)), CL is maximal for pH comprised between 8.5 and 9 and is almost equal to zero for pH superior to 10. This indicates that at vicinity of glass, peroxidase acts effectively as a catalyst for the reactions responsible for luminol CL. This result is in agreement with the fact that peroxidase was already found to catalyze CL at vicinity of glassy carbon electrodes. On the contrary, in the case of Ag and Au (\textbf{Figure V.II}(a)), CL is low at pH 8.5 and is maximal in the pH range comprised between 9 and 11. This indicates that CL generated by the catalytical activity of peroxidase is strongly decreased when the enzyme is fixed on flat metal film which is probably due to a light emission quenching not yet negligible at a distance of around 10 nm from the metal.
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Figure V.11 Effect of pH upon the chemiluminescence of the luminol-H$_2$O$_2$ system catalyzed by peroxidase at vicinity of flat Au and Ag films (a) and glass (b) for different roughness. Inset: CL at different pH on flat Ag films in presence or in absence of peroxidase; from left to right, pH in first row corresponds to 7, 8, 8.5 and 9 and in second row to 10, 11, 11.5 and 12.

Figure V.12 Effect of pH upon the chemiluminescence of the luminol-H$_2$O$_2$ system catalyzed by peroxidase at vicinity of corrugated (cluster-like) Au and Ag films.
Corrugation induces an enhancement of the chemiluminescence at pH 9.

Figure V.12 gives the CL intensity as a function of pH on cluster-like Au and Ag films. The first observation is that, for both metals, CL is systematically greater than in the case of flat films. This shows that nano-corrugation induces a significant CL enhancement at all the pH values studied and not only at pH 8.5 (the pH fixed in previous experiments). Moreover whereas CL presents a maximum at pH 9 for both metals, the signal is much more important for nano-corrugated gold which indicates clearly that it is a better catalyst than corrugated silver. Concerning silver, very striking is that the position of the CL maximum depends on the morphology of the film: whereas in absence of corrugation, CL is maximal at around pH 10 (a range favouring the reactions taking place without help of peroxidase), it is maximal at pH 9 in presence of corrugation. Since pH 9 increases peroxidase activity, this could prove that corrugation enhances preferentially the reactions involving the enzyme. This conclusion is reinforced by the case of gold for which the maximal CL observed is also shifted in the exact range of pH (8.5 – 9) favouring peroxidase-related reactions. In absence of peroxidase, Zhang et al. found that gold particles significantly enhance CL of the luminol-H$_2$O$_2$ system at pH 12 \cite{6}. They suggest that the O-O bond of H$_2$O$_2$ is broken up into double OH’ radicals by virtue of gold catalysis facilitating then the formation of luminol radical L'. Our present experiments confirm this result since at pH 12 (a pH at which peroxidase does not act as a catalyst) they also evidence a CL enhancement induced by corrugation. Indeed luminol CL increases of around one order of magnitude from 10±3 in arbitrary units at vicinity of flat gold (Figure V.13) up to 130±40 near corrugated gold (Figure V.12). However, when peroxidase is grafted on corrugated gold, luminol CL is maximal at pH 9 (the pH enhancing catalysis by peroxidase) and strongly greater than at pH 12: 61300 instead of 130 in the same arbitrary units. The reactions involving peroxidase are then also surface-enhanced but with an enhancement factor of two orders of magnitude (at pH 9, CL increases from 600 at vicinity of flat gold up to 61300 near corrugated gold).

V.2.4 Catalytical mechanism of SECL

To definitely prove that SECL is related to a catalytic effect, three chemically different samples (Au, Ag, AuAg alloys) were studied. AFM images and extinction spectra of the samples are given in Figure V.14. During further immersion in biological media, Ag films were found to remove from the substrates except for deposition at 600°C. To increase Ag adhesion, a thin film of BaTiO$_3$ was intercalated between the quartz substrate and the silver film. This strengthened sufficiently Ag adhesion to achieve satisfactorily further CL experiments while the presence of BaTiO$_3$ induced a red shift of the SPR band (from 530 to
620 nm for the film deposited at 600°C). All the characteristics of the films studied are indicated in Table V.5. The specific area of gold which depends on roughness is evaluated from the average diameter, D, and the height, h, of the clusters. Compared with flat surfaces, the specific area of a rough film increases by a factor of Kh/D where K is a dimensionless coefficient depending on the exact geometry of the clusters and close to 2. The different preparations allowed (i) to vary the distance between the luminol emission peak at 425 nm and the position of the plasmon band in a large extent (from 105 nm for the Ag film prepared at 600°C to 245 nm for the Au one prepared at 200°C) and (ii) to get three chemically different samples (Au, Ag, AuAg alloys) all of them having their plasmon absorption peaking at 620 nm (Figure V.14 right and Figure V.16(b)). The 620 nm lies far from luminol emission (425 nm), so that the low overlapping resulting between SPR and dye luminescence should limit plasmon assistance to CL enhancement. Then, if strong differences are evidenced in CL between all the metals investigated, SECL should clearly originate from catalytic mechanisms.

![AFM images](image)

**Figure V.14** Left, AFM images (area of 500 nm x 500 nm) of “bulk-like” Au (a), AuAg (b) and Ag (c) films prepared at room temperature and “cluster-like” Au (d), AuAg (e) and Ag (f) films prepared at high temperature. Right, extinction of different “cluster-like” films (Ag (up), AuAg (middle) and Au (down)) deposited on glass at 200, 300 and 600°C and
extinction of a “cluster-like” film of Ag deposited on BaTiO3 supporting glass at 600°C (up).

**Table V.5 Characteristics of the samples studied. “Surface area ratio” is the ratio between specific areas of rough and flat films.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temperature (°C)</th>
<th>SPR position (nm)</th>
<th>SPR HWHM (nm)</th>
<th>SPR intensity</th>
<th>Roughness (nm)</th>
<th>Particle size (nm)</th>
<th>Surface area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au film</td>
<td>30</td>
<td>No peak</td>
<td>No peak</td>
<td>No peak</td>
<td>0.3</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>620</td>
<td>72</td>
<td>0.42</td>
<td>1.9</td>
<td>~35</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>AuAg film</td>
<td>30</td>
<td>No peak</td>
<td>No peak</td>
<td>No peak</td>
<td>0.4</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>620</td>
<td>150</td>
<td>0.46</td>
<td>4.0</td>
<td>27</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>582</td>
<td>80</td>
<td>0.52</td>
<td>4.3</td>
<td>30</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>576</td>
<td>60</td>
<td>0.61</td>
<td>4.4</td>
<td>32</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Ag film</td>
<td>30</td>
<td>No peak</td>
<td>No peak</td>
<td>No peak</td>
<td>0.3</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>530</td>
<td>123</td>
<td>0.84</td>
<td>3.1</td>
<td>35</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Ag film on BaTiO3</td>
<td>600</td>
<td>620</td>
<td>130</td>
<td>0.70</td>
<td>3.6</td>
<td>37</td>
<td>1.35 ± 0.2</td>
</tr>
</tbody>
</table>

The efficiency of peptides grafting on the different films was evaluated by X-ray Photoelectron Spectroscopy (XPS) ([Figure V.15](#)) that allowed determining the surface atomic ratios: S/metal, N/metal, N/S and Au/Ag ([Table V.6](#)). One can assume that the metal depth analyzed in XPS is given by the product: $\lambda$ [metal] where $\lambda$ is the inelastic mean free path of the photoelectrons ($\approx 5$ nm for Au and $\approx 10$ nm for Ag) and [metal] is the atomic metal concentration found by XPS. The peptide density can then be easily obtained from the metal density (59.0 atoms/nm$^3$ for Au and 58.7 atoms/nm$^3$ for Ag) taking into account that each peptide contains 4 S atoms. Whatever the film, the peptide density is comprised between 1 and 4 peptide/nm$^2$. This density is lower than that normally obtained for mono-thiolate grafting on a flat Au (111) surface which is of 5 peptides/nm$^2$. This can be easily explained by the higher steric hindrance of the tri-thiolated peptides used here.
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Figure V.15 Spectra XPS of N1s on gold (a) and silver (c) films before and after the deposition of peptides (P23), and S 2p on gold films (b) and silver (d) films before and after the deposition of peptides (P5).
### Table V.6 Atomic ratios (as determined by XPS), metal concentration ([metal]), peptide density, and average distance between adjacent peptides.

<table>
<thead>
<tr>
<th></th>
<th>N/S</th>
<th>N metal</th>
<th>S metal</th>
<th>Au/Ag</th>
<th>[metal]</th>
<th>Peptide density (peptide/nm²)</th>
<th>Average distance between two peptides (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat Au P11</td>
<td>3.9 ± 0.8</td>
<td>0.35 ± 0.07</td>
<td>0.09 ± 0.02</td>
<td>_</td>
<td>0.20</td>
<td>1.3 ± 0.4</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>Rough Au P5</td>
<td>2.25 ± 0.4</td>
<td>0.28 ± 0.06</td>
<td>0.13 ± 0.03</td>
<td>_</td>
<td>0.21</td>
<td>2.0 ± 0.6</td>
<td>0.7 ± 0.02</td>
</tr>
<tr>
<td>Rough Au P11</td>
<td>4.5 ± 0.8</td>
<td>0.45 ± 0.09</td>
<td>0.11 ± 0.02</td>
<td>_</td>
<td>0.20</td>
<td>1.6 ± 0.5</td>
<td>0.8 ± 0.02</td>
</tr>
<tr>
<td>Rough Au P17</td>
<td>7.25 ± 1.2</td>
<td>0.49 ± 0.1</td>
<td>0.08 ± 0.02</td>
<td>_</td>
<td>0.18</td>
<td>1.1 ± 0.4</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>Rough Au P23</td>
<td>9.75 ± 1.4</td>
<td>0.49 ± 0.1</td>
<td>0.07 ± 0.02</td>
<td>_</td>
<td>0.17</td>
<td>0.9 ± 0.3</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>Rough Ag P5</td>
<td>2.2 ± 0.4</td>
<td>0.37 ± 0.08</td>
<td>0.27 ± 0.06</td>
<td>_</td>
<td>0.10</td>
<td>4.0 ± 1.2</td>
<td>0.5 ± 0.02</td>
</tr>
<tr>
<td>Rough Ag P23</td>
<td>7.2 ± 1.4</td>
<td>1.01 ± 0.2</td>
<td>0.14 ± 0.03</td>
<td>_</td>
<td>0.09</td>
<td>1.9 ± 0.6</td>
<td>0.7 ± 0.02</td>
</tr>
<tr>
<td>Rough AuAg P5</td>
<td>2.4 ± 0.4</td>
<td>0.34 ± 0.07</td>
<td>0.19 ± 0.03</td>
<td>1.1</td>
<td>0.15</td>
<td>3.1 ± 0.9</td>
<td>0.6 ± 0.02</td>
</tr>
</tbody>
</table>

Different conclusions can be drawn from the values given in Table V.6. First the grafting density on gold is the same whatever the nature of the film, bulk-like or cluster-like (for P11 grafting, the density is of 1.3 peptides/nm² on a flat surface and of 1.6 peptides/nm² on a rough one). Second, as expected from an increasing hindrance, the peptide density slightly decreases with the peptide length: from 2 peptides/nm² for P5 to 0.9 peptides/nm² for P23 when grafting on a gold film made at 600°C. Third, the grafting density is higher on films containing silver than those made from pure gold.

XPS was also performed to characterize the grafting of streptavidin labeled peroxidase on biotinylated peptides. After reaction of the 6-10 nm sized peroxidase on the tri-thiolated peptides, metal becomes quasi undetectable whatever the film and the peptide used. This indicates that for all films, peroxidase coats continuously the surface. This result could be already inferred from the peptides density evaluated by XPS. Due to the high affinity of streptavidin for biotin and to the fact that a biotinylated site is available at less every nm whereas streptavidin labeled peroxidase has a size of 6-10 nm (see Table V.6), all the peptide

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densities are indeed sufficient to ensure an homogeneous peroxidase coating. In accordance with literature, this peroxidase coating induces significant modifications in extinction spectra (Table V.7): a red-shift, a broadening and a height increase of the plasmon peak. The red-shift slightly increases with the peptide length (from 24 nm for P5 to 30 nm for P23 when grafting on a gold film made at 600°C) and is greater for silver than for gold (44 nm instead of 29 nm when peroxidase is grafted via P11). The sensitivity of SPR to changes in the surrounding environment of metal depends only slightly on the nature of the metal. Then, the latter observation could be explained by the greater density of peptides grafted in the case of Ag.

Table V.7 Evolution of extinction spectra with biological coating for three films: Au, AuAg and Ag/BaTiO₃. Coating consists of peroxidase labelled streptavidin grafted on metal via different peptides: P5, P11, P17 and P23.

<table>
<thead>
<tr>
<th>Type of coating</th>
<th>SPR position (nm)</th>
<th>SPR HWHM (nm)</th>
<th>SPR intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>No coating</td>
<td>620</td>
<td>72</td>
<td>0.42</td>
</tr>
<tr>
<td>P5</td>
<td>644</td>
<td>83</td>
<td>0.46</td>
</tr>
<tr>
<td>P11</td>
<td>649</td>
<td>83</td>
<td>0.45</td>
</tr>
<tr>
<td>P17</td>
<td>652</td>
<td>85</td>
<td>0.52</td>
</tr>
<tr>
<td>P23</td>
<td>650</td>
<td>87</td>
<td>0.49</td>
</tr>
<tr>
<td>No coating</td>
<td>620</td>
<td>150</td>
<td>0.46</td>
</tr>
<tr>
<td>P11</td>
<td>657</td>
<td>169</td>
<td>0.53</td>
</tr>
<tr>
<td>No coating</td>
<td>620</td>
<td>130</td>
<td>0.70</td>
</tr>
<tr>
<td>P11</td>
<td>664</td>
<td>150</td>
<td>0.79</td>
</tr>
</tbody>
</table>

The behavior of luminol CL at vicinity of cluster-like Ag films completely differs from that observed at vicinity of Au or AuAg ones (Figure V.16(a)). Whereas corrugation leads to an enhancement of more than one order of magnitude for Au and AuAg, it leads to only an increase comprised between 50 and 100% for Ag. This (relatively) low increase is consistent with that already reported by Aslan et al. who studied differently coloured CL reagents [29]. The difference in the enhancement can not be attributed to a deficit in the peptide densities of Ag substrates since the amount of polypeptides grafted is greater for silver than for gold. It definitely then indicates that other mechanisms than plasmon-assisted processes should be
involved in SECL and as stated by Zhang et al.\cite{6}, the nature of the main mechanisms responsible for SECL should be catalytic.

Figure V.16 (a) luminol-H$_2$O$_2$ CL induced by peroxidase on cluster-like Au, Ag and Au-Ag films prepared at different temperatures with different metal/peroxidase distances. (b) extinction spectra of Au, Ag and AuAg films.

CL is consecutive to luminol oxidation into a luminol radical. Two main mechanisms for catalysing this oxidation can be proposed. The first mechanism can be inferred from the apparent contradiction that catalysis involves contact interaction occurring at distances up to 1 nm whereas the CL enhancement is evidenced here for metal/peroxidase distance largely
greater (around 5 nm). Since the oxidation is known to process in two steps: \(^{[22]}\) one involving peroxidase (and then taking place in close contact with it) and the other involving the presence of an oxygen-related radical (and then not necessarily required to append at close vicinity of peroxidase), this is this latter step which is most certainly catalysed by the presence of corrugated gold. \(^{[30]}\) According to the reactions (R), the catalytic mechanisms involving peroxidase require the presence of hydrogen peroxide. It is then possible that metal catalysis could arise from an enhanced electron transfer from metal clusters to adsorbed \(\text{H}_2\text{O}_2\). This particle-mediated transfer, \(^{[18]}\) permits to produce some key radicals favouring the formation of complexes containing peroxidase and/or that of luminol radical in presence of these complexes. This interpretation is based on several reports evidencing the formation of active oxygen-containing reactant intermediates such as \(\text{OH}^+\) or \(\text{O}_2^−\) when gold particles are used as catalysts. \(^{[31]}\) The second mechanism, acting in conjunction with the first one, could arise from a decrease of the redox potential of luminol at vicinity of corrugated gold. Such a shift which facilitates luminol oxidation in presence of catalysing peroxidase was already evidenced by our team when luminol is directly fixed on gold cluster. \(^{[32]}\) In any case, the catalytic enhancement arises from the modification of thermodynamic properties at vicinity of highly curved surfaces \(^{[33]}\) and is expected to increase with roughness for all the metals investigated here. This correlation was already observed in the case of gold cluster \(^{[34]}\) for which CL increases by 25\% when roughness increases from 1.3 to 1.9 nm. We also verified it for AuAg alloys for which CL increases of more than 50\% for a roughness increase from 4.0 to 4.4 nm and even for Ag for which an increase of roughness from 3.1 to 3.6 nm leading to a CL increase of around 30\%.

Figure V.17 Relationship between the CL and SPR for metal families that differ in their...
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composition: Au, Ag and AuAg alloys. Within a same family the different points correspond to different corrugations (and for Au to different peptide grafting).

A correlation between SPR intensity and CL enhancement has already been pointed out in literature\textsuperscript{[6]}. Figure V.17 which summarizes all the results that we have obtained shows that, in fact, such correlations can be found within each metal family (Au, Ag or Au/Ag) but can not lead to an universal rule that would be valid regardless the nature of the metal. The reason for which such a correlation can be established within each kind of metal is that SPR intensity and SECL have a similar evolution with roughness. Roughness increases the number of localized electrons responsible for strong Mie resonances. It increases also the film curvatures responsible for an increased catalytic activity. Then, whereas LSPR intensity is an indicator of the number of metal electrons liable to catalyse CL\textsuperscript{[35]}, the efficiency of the catalysis depends on the chemical nature of the metal.

V.3 Conclusions

In this chapter, a system of biological detection based on SECL was studied. Metal thin films with nanometer-scale roughness were fabricated by PLD technique to demonstrate the phenomenon of SECL: when the gold films support a catalyst (the peroxidase) of luminol CL, a corrugation of the films generates an emission enhancement of the luminol brought to proximity.

In order to better understand this phenomenon, several parameters were investigated, such as the distance between films and peroxidases, nanostructure of thin films, pH value of luminol solution and chemical nature of thin films. It was found that luminol CL which occurs at peroxidase vicinity depends on not only the distance between films and peroxidases but also the metal nanostructure. When peroxidase is attached on a bulklike film, CL increase monotonously with the distance because of a decrease of the light emission quenching by metal; when peroxidase is attached on a clusterlike film, CL undergoes a complex variation with the metal/catalyst distance evidencing a competition between quenching process and a nanostructure-induced catalysis enhancement. Hexagonal Au particles arrays were also fabricated to further investigate the influence of nanostructure upon CL. For different metal/peroxidase distances, Au particles arrays 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 15nm are efficient for catalysis. CL enhancement induced by nanoscaled-corrugation of metal was found to reach a maximum at a optimal pH of 9, a value belonging to the range which
favors the reactions catalysed by peroxidase.

After a large number of samples were studied, in which the chemical nature of the film and the position of the SPR peak were independently varied, a plasmon-assisted processes to be eliminated as the main mechanisms responsible for SECL. The CL enhancement was related to catalytic mechanisms, the efficiency of which is governed by a key parameter, the roughness. Nanoscale-corrugated gold appears as a better catalyst than silver for SECL.
Surface-enhanced chemiluminescence by rough metal films

References:

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VI  General conclusions
The overall goal of the thesis is to develop two new systems based gold nanostructure for biological detection. The first system focuses on the possibility of multimodal detection using LSPR of the nano-gold substrate and the luminescence of labelling nanoparticles; the second one is based on the catalytic properties of gold nanostructure: SECL.

In order to study the two system of biological detection, three techniques were used to fabricate nano-structure substrates, which are PLD, NSL and EBL techniques. Bulk-like and cluster-like thin films (Au, Ag, and Au-Ag alloys) were prepared by PLD technique by controlling two main parameters: substrate temperature and film thickness during deposition. Gold nanoparticle arrays were obtained by NSL using a single layer or double-layer polystyrene nanosphere mask. Another method of lithography fabricating gold nanoparticle arrays is EBL, which permits to serially obtain array patterns with different particle’s shape, size, and interparticle space.

To investigate a multimodal detection system using their LSPR and the luminescence of labelling nanoparticle, core-shell particles which consist of a gadolinium oxide core encapsulated in a polysiloxane shell (Gd$_2$O$_3$/SiO$_x$) containing rhodamine were deposited to the gold substrates to realize the luminescent detection. Tb-doped Gd$_2$O$_3$ core particles have been prepared using the polyol method and in-situ luminescent spectra have been investigated to follow up the process of synthesis. Compared with the classical synthesis, the synthesis was optimized by decreasing the temperature of NaOH addition from 140°C to room temperature. As soon as NaOH was added in the precursor solution, Gd$_2$O$_3$:Tb$^{3+}$ particles could be formed whatever the manner of adding NaOH (on-off addition or progressively addition) at room temperature. Whereas for the latter manner, the particles were nucleated in line and had a little bigger size (1.5nm). For both cases after being annealed at 160 °C, the little particles could grow up. However, the annealing was more efficient for progressive addition of NaOH, since the average size of particles reached 3.5nm after annealing.

The size induced modifications of the electronic structure of the nanoparticles were investigated by EELS. According to the spectra of O K edge for particles size from 1.1nm to 4nm, a broad peak at 540.5 eV appeared as a result of mixing of the O p with the delocalized Gd 5d and 6s states. Whereas for particles size smaller than 2.7nm, another peak attributed to the mixing of the O p with the Gd 4f was observed to locate between 530-540 eV. The intensity as well as the energy of the peak decreased with the increase of particle size and even disappeared for particle size above 2.7nm. These observed changes are thus a signature of the modification of the Gd-O chemical bond as a function of the particles size. This result is consistent with the spectra of Gd N$_{4.5}$, in which multiplet effects arising from the strong overlap between the Gd 4d and localized 4f radial wave functions give rise to the narrow pre-edge peaks below 140 eV and the broad structure around 145 eV. The pre-edge peaks are
broader for particles smaller than 2.7nm and shifted by 0.5 eV towards higher energies compared to those obtained from particles above 2.7nm. The results demonstrate a charge transfer from Gd to O in Gd₂O₃ resulted in a delocalization of a part of the f electrons via hybridization with the valence band.

Core-shell particles were obtained by coating a polysiloxane shell to Gd₂O₃ core, for which TEOS and APTES were chosen as two precursors of shell. Fluorophores are added in the polysiloxane layer by being mixed with APTES in dimethylsulfoxide (DMSO). several kinds of polysiloxane shell with different thickness were coated to the same gadolinium oxide core by modifying the quantity of precursors added, which aims to follow up the growth of the polysiloxane shell according to the composition of the encapsulating solution. Compared with the estimated result, the experimental result shows that only a small part (9.75%-16.6%) of encapsulating solution took part in the elaboration of polysiloxane shell. The morphology of core-shell particles were visualized by TEM images.

With these two systems so developed (gold substrates, core-shell particles), we obtained the following results:

- the biotin-streptavidin binding can be detected by three types of techniques: the LSPR shift and the two kinds of luminescence of the particle, the organic one of the shell encapsulating dyes as RBITC or FITC and the inorganic one related to Tb³⁺. To evidence the latter a simultaneous optimization of the detection system (laser) and of annealing (reducing conditions) was necessary. That opens promising routes for multi-labelling strategies by crossing organic and inorganic optical signals.

- attaching a particle to the biomolecule to detect allows to eliminate upon annealing all the undesired molecules that skew the assays and so increases the reliability of detection.

- Attaching a particle that ballasts the protein also enhances its binding kinetics and increases the localized surface Plasmon resonance shift that detects the binding.

Another system of biological detection based on SECL was studied. Metal thin films with nanometer-scale roughness were fabricated by PLD technique to demonstrate the phenomenon of SECL: when the gold films support a catalyst (the peroxidase) of luminol CL, a corrugation of the films generates an emission enhancement of the luminol brought to proximity.

To better understand this phenomenon, several parameters were investigated, such as the distance between films and peroxidases, nanostucture of thin films, pH value of luminol solution and chemical nature of thin films. It was found that luminol CL which occurs at
peroxidase vicinity depends on not only the distance between films and peroxidases but also the metal nanostructure. When peroxidase is attached on a bulk-like film, CL increase monotonously with the distance because of a decrease of the light emission quenching by metal; when peroxidase is attached on a clusterlike film, CL undergoes a complex variation with the metal/catalyst distance evidencing a competition between quenching process and a nanostructure-induced catalysis enhancement. Hexagonal Au particles arrays were also fabricated to further investigate the influence of nanostructure upon CL. For different metal/peroxidase distances, Au particles arrays 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 15nm are efficient for catalysis. CL enhancement induced by nanoscaled-corrugation of metal was found to reach a maximum at an optimal pH of 9, a value belonging to the range which favors the reactions catalysed by peroxidase.

After a large number of samples were studied, in which the chemical nature of the film and the position of the SPR peak were independently varied, a plasmon-assisted processes to be eliminated as the main mechanisms responsible for SECL. The CL enhancement was related to catalytic mechanisms, the efficiency of which is governed by a key parameter, the roughness. Nanosacle-corrugated gold appears as a better catalyst than silver for SECL.
Appendix
X-ray Photoelectron Spectroscopy (XPS)

In our work, XPS analysis of the gold films before and after deposited biomolecules such as peptides was carried out at the "Institut de Recherche sur la Catalyse" (IRC) with a VG Scientific ESCA LAB 200 R using a 220 W Al K radiation (1486.6 eV). Spectra relative to C1s, O1s, N1s, S2p, Si2s, Au4f7/2 and Ag3d photoelectron peaks were measured at binding energies around respectively 284.6, 531.0, 399.4, 162.8, 153.3, 83.7 and 367.9eV. The spot size on sample is of 400x1000m$^2$. The areas of elements' peaks allows to calculate the atomic ratio according to the formula (as an example of C and N)

\[
\frac{C}{N} = \frac{kn \ area(C)}{kc \ area(N)}
\]  \hspace{1cm} (1)

where kc and kn are the C and N sensitivity factors.

Sensitivity factors contain the photoemission cross sections as approximated from a Hartree-Slater atomic model. With ESCA LAB 200 R, The factors are corrected by a factor proportional to ($E_{kin}$) 0.1. Attenuation from the free electron mean path and from the transfer function of the spectrometer are then accounted for. As a consequence from all the above approximations, absolute uncertainties in XPS quantitative analysis are generally of the order of magnitude of $\pm 10\%$
Figure I. Spectra XPS of C1s on gold films before (a) and after (b) the deposition of peptides.
Table I: XPS results obtained from gold films before and after the deposition of peptides.

<table>
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<tr>
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<th>number</th>
<th>Area</th>
<th>FWHM (eV)</th>
<th>Energy (eV)</th>
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<tr>
<td></td>
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<td>284.5</td>
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</table>

(exp.is the experimental value; theo.is the theoretical value. FWHM is the full width at half maximum of the peak in XPS.)

**Photon Correlation Spectroscopy (PCS)**

PCS (also known as Dynamic Light Scattering) is a technique used to determine the size distribution of particles in solution. A time-dependant fluctuation in scattering intensity can be observed when a laser hits particles in solution due to the Brownian motion of these particles in solution. As illustrated in figure II, more little the particles, more rapidly they move.

![Figure II. Brownian motion of (a) small (b) big particles in solution.](image)

The speed of particles’ motion is defined by z-averaged translational diffusion coefficient $D$ that can be calculated into the hydrodynamic radius for a particle through the
Stokes-Einstein equation:

\[ d(H) = \frac{KT}{3\pi\eta D} \]  

Where \( d(H) \): hydrodynamic diameter

D: Translational diffusion coefficient
\( \eta \): Viscosity
T: Temperature
K: Boltzmann constant.

Translational diffusion coefficient D can be transformed into mathmatique value by an autocorrelation function:

\[ G(t) = \sum c_i \exp(-\gamma_i t) \]  

Where \( t \): Sampling time

\( c_i \): Diffusion intensity
\( \gamma = Dq^2 \)
\( q \): wave vector \( q = \frac{4\pi n}{\lambda}(\sin \alpha / 2) \)

and \( n \): Refraction index of suspension
\( \lambda \): Wave length of laser
\( \alpha \): Angle between laser and detector

In our work, PCS are obtained by a Zetasizer 3000HS (Malvern Instruments), and a photomultiplicator as the detector is placed perpendicularly to the laser considering that the dispersion of diffusing light from small particles is isotropic.

**Nuclear Magnetic Resonence (NMR)**

Nuclei have positive charges and those with an odd number of protons or neutrons behave spinning. Nuclei that are charged and spin have magnetic moment and produce magnetic field, which makes them act as tiny bar magnets oriented along the spin rotation axis (figurelll). If the small magnets are put in the field of a much larger magnet \( B_0 \), its orientation will no longer be random. There will be one most probable orientation that is parallel to the external magnetic field to align the spinning nuclei. However, if the tiny magnet is oriented precisely
180° in the opposite direction, that position could also be maintained. The most favorable orientation is called low-energy state and the less favorable orientation is the high-energy state. Any individual nuclear spin of a nucleus with spin quantum number $I = \frac{1}{2}$ is in one of the two states, which is a quantum mechanical requirement.

**Figure III:** The charged nucleus rotating with angular frequency $\omega$ creates a magnetic field $B$ and is equivalent to a small bar magnet whose axis is coincident with the spin rotation axis.

The small nuclear magnet may spontaneously "flip" from one orientation (energy state) to the other as the nucleus sits in the large magnetic field. Resonant absorption will occur when electromagnetic radiation of the correct frequency (radiofrequency (RF) pulses) to match this energy difference of the two nuclear spin orientations is applied. The absorption of energy by the nuclear spins causes transitions from higher to lower energy as well as from lower to higher energy. This two-way flipping is a hallmark of the resonance process.

The energy required to induce flipping and obtain an NMR signal is just the energy difference between the two nuclear orientations and depends on the strength of the magnetic field $B_0$ in which the nucleus is placed:

$$\Delta E = \gamma h B_0/2\pi \quad (4)$$

Where $h$ is Planck's constant ($6.63 \times 10^{-27}$ erg sec). The Bohr condition ($\Delta E = h\nu$) enables the frequency $\nu$ of the nuclear transition to be written as

$$\nu = \gamma B_0/2\pi \quad (5)$$

Equation (4) is often referred to as the Larmor equation, and $\omega_0 = 2\pi\nu_0$ is the angular Larmor resonance frequency. The gyromagnetic ratio $\gamma$ is a constant for any particular type of nucleus.
of nucleus and is directly proportional to the strength of the tiny nuclear magnet.

Relaxation processes eventually return the spin system to thermal equilibrium, which occurs in the absence of any further perturbing RF pulses. Two principal relaxation processes are termed $T_1$ and $T_2$ relaxation respectively. $T_1$ (longitudinal relaxation time) is the decay constant for the recovery of the $z$ component of the nuclear spin magnetization, $M_z$, towards its thermal equilibrium value, If $M$ has been tilted into the $xy$ plane, then $M_z(0) = 0$ and the recovery is simply:

$$M_z(t) = M_0 (1 - e^{-t/T_1})$$

(6)

$T_1$ is the time that magnetization recovers to 63% of its initial value. Owing to the fact that $T_1$ relaxation involves an interaction (energy exchange) with the surroundings (the lattice), then $T_1$ is also called spin-lattice relaxation time. $T_2$ (transverse relaxation time) is the decay constant for the component of $M$ perpendicular to $B_0$, designated $M_{xy}$:

$$M_{xy}(t) = M_0 e^{-t/T_2}$$

(7)

$T_2$ is the time that transverse magnetization vector drops to 37% of its original magnitude. $T_2$ corresponds to a decoherence of the transverse nuclear spin magnetization. Random fluctuations of the local magnetic field lead to random variations in the instantaneous NMR precession frequency of different spins. As a result, the initial phase coherence of the nuclear spins is lost, until eventually the phases are disordered and there is no net $xy$ magnetization. Because $T_2$ relaxation involves only the phases of other nuclear spins it is often called "spin-spin" relaxation. The diminution of the transverse magnetization component usually occurs faster than the regrowth of the longitudinal component, and $T_2$ is therefore always shorter than $T_1$.

In our work, nuclei relaxation times were recorded by a MiniSpec mq60 from Bruker (Germany), a MiniSpec Contrast Agent Analyzer. $T_1$ and $T_2$ were measured during the synthesis of core and process of encapsulation.