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Ethylene Diamine Functionalized Citrate-Capped Gold Nanoparticles for Metal-Enhanced Bioluminescence

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Abstract. Metal-enhanced bioluminescence (MEB) is a complex photophysical event which manifests itself in manifold luminescence enhancement and depends on many parameters. The parameters include the distance between the luminescent source and the nanomaterial surface, the size and shape of nanoparticles, localized surface plasmon resonance (LSPR) peaks of the nanomaterial and the dielectric constant of the surrounding medium. Studying distance-dependent MEB in the presence of linkers of specified length may provide new insights into luminescence enhancement. In this regard, the present investigation is aimed to understand the role of ethylene diamine as a potential linker in model systems for distance-dependent metal-enhanced bioluminescence (MEB) with gold nanoparticles (AuNPs). Four different types of AuNPs (AuNP1, AuNP2, AuNP3, AuNP4) were synthesized by varying the trisodium citrate (TC) and silver nitrate ($AgNO_3$) concentrations with maximum absorbance values of 0.99, 1.24, 1.21 and 1.38 respectively and the corresponding LSPR peaks (λ_{max}) of 520 nm, 535 nm, 525 nm, and 525 nm. Luminescence enhancement up to 1.44-fold was observed when ethylene diamine (ED) was used as a linker in the presence of 1-N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), ATP and AuNP1. The sample consisting of FMN, EDC and AuNP1 showed 1.3-fold luminescence enhancement. It was noted that AuNPs synthesized using $AgNO_3$ as an additional component did not enhance luminescence in all the investigations. The suggested technique of using linkers of predetermined lengths may also prove fairly effective for studying other parameters which can influence MEB and cause sensitivity enhancement of luminescence-based biosensors.

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Покрытые цитратной оболочкой и функционализированные этилендиамином наночастицы золота для усиления биолюминесценции металлом

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Аннотация. Металл – усиленная биолюминесценция (МЕВ) представляет собой сложное фотофизическое событие, проявляющееся в виде многократного усиления люминесценции и зависящее от множества параметров. К этим параметрам относится расстояние между источником люминесценции и поверхностью наноматериала, размер и форма наночастиц, а также пики плазмонного резонанса локализованной поверхности (LSPR) наноматериала и диэлектрическая проницаемость окружающей среды. Изучение расстояния между источником люминесценции и поверхностью наноматериала с использованием связующих агентов (линкеров) заданной длины может дать новое понимание эффекта усиления люминесценции. Поэтому целью настоящего исследования была проверка возможности использования этилендиамина в качестве потенциального линкера для дистанционно-зависимой МЕВ с наночастицами золота (AuNPs). Путем варьирования концентраций тринатрийцитрата (ТС) и нитрата серебра ($AgNO_3$) были синтезированы четыре типа наночастиц золота (AuNP1, AuNP2, AuNP3, AuNP4) с максимумами поглощений 0,99; 1,24; 1,21 и 1,38 при λ_{max} 520, 535, 525 и 525 нм. Усиление люминесценции в 1,44 раза наблюдалось при использовании этилендиамина (ED) в качестве линкера в присутствии 1-этил-3-(3-диметиламинопропил)карбодиимида (EDC), АТР и AuNP1. В образце, состоящем из флавинмононуклеотида (FMN), EDC и AuNP1, было зафиксировано усиление люминесценции в 1,3 раза. Использование $AgNO_3$ в качестве дополнительного компонента при синтезе AuNPs не приводит к усилению уровня люминесценции. Предложенный подход с применением линкеров заданной длины может быть полезным для изучения других параметров, влияющих на МЕВ и приводящих к увеличению чувствительности люминесцентных биосенсоров.

Ключевые слова: усиление биолюминесценции металлом, этилендиамин, карбодиимиды, аденозин 5'-трифосфат, флавинмононуклеотид.

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Introduction

Metal-enhanced bioluminescence (MEB) is an intriguing photophysical effect that takes place on a nanometal surface and depends upon multiple parameters (Geddes, Geddes, 2012; Geddes, 2014; Ranjan et al., 2017). These parameters include the distance between the source of light and plasmon-induced metallic surface (generally ≤10 nm), the wavelength and intensity of the incident light, the nature of nanomaterial (shape, size, metal type) and the dielectric constant of the surrounding medium. For this reason, determining the optimal conditions for observing luminescence enhancement is a highly complex task. Nevertheless, there are a few scientific reports which discuss MEB. The paper by Golberg et al. (2014) describes bioluminescence enhancement (up to 2-fold) by silver nanoparticles on a polystyrene microtiter plate surface placed in vicinity to a whole-cell bioreporter. The distance ($d_{L\text{-}NP}$) between the nano-metal surface and the light source (viable bioluminescent bacteria) was presumed to be less than 10 nm. Another similar MEB study was carried out using gold-silver colloids in association with bioluminescent sources viz. *Photobacterium leiognathi*, firefly luciferase (FLuc) and bacterial luciferase (BLuc). The achieved enhancement of bioluminescence was 250% for bacterial cells, 95% for BLuc and 52% for FLuc (Abhijith et al., 2014). This study was carried out without considering $d_{L\text{-}NP}$. Use of silver island films in bioluminescence enhancement has also been previously reported (Eltzov et al., 2009) and $d_{L\text{-}NP}$ was intentionally less than 10 nm. In this case, the excited states of the bioluminescent intermediates can non-radiatively induce mirror dipoles on the metallic surface thus inducing the surface plasmons to radiate coupled quanta. According to Du et al. (2014) bioluminescent protein nanocapsules (BPN) in close proximity to gold nanocrystals (GNC) can result in ca. 10-fold enhancement of bioluminescence intensity. In this research, BPN was constructed using chemically modified horse-radish peroxidase (HRP) and GNC.

These methods for bioluminescence enhancement provide ample opportunity to increase the sensitivity of bioassay systems. However, an appropriate linker of a uniform size has not been previously used for observing MEB. In the present research, ethylene diamine (ED) was studied as a potential linker between a bioluminescent source and gold nanoparticles (AuNPs) in MEB. Adenosine 5'-triphosphate (ATP) and flavin mononucleotide (FMN) were used as prime substrates for bioluminescent emission in this study.

Materials and methods

Materials

A vial of the lyophilized preparation contained 0.5 mg bacterial luciferase (EC 1.14.14.3) from the recombinant strain, *Escherichia coli* and 0.15 U of NADH:FMN–oxidoreductase (EC 1.5.1.29, *Vibrio fischeri*) obtained from the Laboratory of Nanobiotechnology and Bioluminescence, Institute of Biophysics, Krasnoyarsk. Mutant

firefly luciferase (FLuc: EC 1.13.12.7) from *Luciola mingrelica* (FLuc) and D-luciferin were the products of Lumtek, Moscow, Russia. Adenosine 5'-triphosphate (ATP), tris(hydroxymethyl)aminomethane (Tris), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and ethylene diamine (ED) were obtained from Merck KGaA, Darmstadt, Germany. Reduced nicotinamide adenine dinucleotide (NADH), tetradecanal and flavin mononucleotide (FMN) were the products of Serva, Germany. Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$) was obtained from Alfa Aesar (ThermoFisher Inc., Karlsruhe, Germany); trisodium citrate was purchased from PanReac AppliChem GmbH, Germany. Silver nitrate ($AgNO_3$) was obtained from JSC Ural Chemical Reagents Plant, Sverdlovsk Region, Russia. Nitric acid (HNO_3) and hydrochloric acid (HCl) were purchased from Giredmet, Moscow, Russia. Glycerol and magnetic beads were purchased locally. Disodium hydrogen phosphate and monosodium dihydrogen phosphate were purchased from Medigen, Novosibirsk, Russia. Glacial acetic acid was obtained from Chemicalsnab, Ufa, Russia.

Synthesis of gold nanoparticles and basic characterization

Glassware viz. a three-neck round bottom flask, glass stoppers, beakers, pipettes and a reflux condenser were washed thoroughly and immersed in aqua regia (mixture of HNO_3 and HCl in the molar ratio 1:3) to eliminate any traces of impurities followed by rinsing with double distilled water. The glassware was dried using a hot air oven until further use. The stock solution of $HAuCl_4 \cdot 3H_2O$ (0.1 M) was prepared by dissolving 0.393 g of $HAuCl_4 \cdot 3H_2O$ in ultrapure water to obtain 10 mL of aqueous solution and kept at 4 °C in the dark; 0.1% $AgNO_3$ aqueous stock solution was prepared by dissolving 2 mg of $AgNO_3$ in 2 mL of ultrapure water and used immediately.

Four different types of AuNPs (AuNP1, AuNP2, AuNP3 and AuNP4) were prepared using the method described previously (Abhijith et al., 2014; Ranjan et al., 2018) with minor modifications. In brief, 145 µL $HAuCl_4 \cdot 3H_2O$ (0.1 M) and 20 mg trisodium citrate (TC) were used to synthesize all AuNPs types. AuNP2, AuNP3 and AuNP4 contained 45µL, 90 µL and 135 µL of $AgNO_3$ (0.1%) respectively. The reaction mixture was made up to the volume of 10 mL using ultrapure water and incubated for 10 minutes in the dark. Meanwhile, 40 mL of ultrapure water was allowed to boil in a 3-neck round bottom flask under reflux using a magnetic stirrer with a heating facility (RH basic 2, IKA, Germany). The flask was partially immersed in a glycerol filled glass jar, where glycerol served as a heat transfer medium.

Then the reaction mixture was transferred to the 3-neck round bottom flask under continuous stirring using a magnetic bead and allowed to react for 15 minutes. The final concentrations of the reactants were: 1.54 mM TC and 0.29 mM $HAuCl_4 \cdot 3H_2O$ in AuNP1; 1.54 mM TC, 0.29 mM $HAuCl_4 \cdot 3H_2O$ and 5.3 µM $AgNO_3$ in AuNP2; 1.54 mM TC, 0.29 mM $HAuCl_4 \cdot 3H_2O$ and 10.6 µM $AgNO_3$ in AuNP3; 1.54 mM TC, 0.29 mM $HAuCl_4 \cdot 3H_2O$ and 15.9 µM $AgNO_3$ in AuNP4. After 15 minutes, the solution was allowed to cool at room temperature. The synthesized AuNPs were transferred to glass vials and an aliquot of each AuNPs was analyzed for its spectrophotometric parameters (absorbance, λ_{max}) using UV-Vis spectrophotometer (Cary 60, Agilent technologies, USA).

Optimization of EDC and ED concentrations

Optimization of EDC and ED concentrations was performed to prevent AuNPs aggregation.

20 µL EDC at concentration ≥10 µM were added to 280 µL of synthesized AuNPs and the color change from red to purple-blue was observed as an indicator of AuNP aggregation. Similarly, ED (≥10 µM) was incubated with 280 µL of AuNPs to determine the optimal concentration that prevented aggregation.

Co-incubation of ED, ATP and AuNPs in the presence of EDC

Four incubation schemes were designed in which the first experiment set-up contained 470 µL deionized water and 30 µL ATP solution; the second experiment set-up involved incubation of individually synthesized AuNPs (410 µL) with 30 µL of ATP solution. The final volume was made up to 500 µL with water. The third experiment set-up included 410 µL AuNPs, 30 µL ATP, 30 µL EDC and 30 µL H_2O; the fourth experiment included 410 µL AuNPs, 30 µL ATP, 30 µL EDC and 30 µL ED. The final concentrations of reagents in the solutions were 100 µM ATP, 100 µM EDC and 10 µM ED. The samples were incubated at 30°C for 60 minutes followed by performing the firefly luciferase bioluminescence assay. The pre-reaction mixture for the bioluminescent assay contained 300 µL tris-acetate buffer (pH, 7.8; 100 mM), 10 µL firefly luciferase (ca. 2 ng FLuc) and 10 µL luciferin (1 µg mL^{-1}). Bioluminescent reaction was initiated by addition of incubated samples (10 µL) to the pre-reaction mixture. Luminescence measurements (n=3) were performed for 60 seconds using a luminometer (Glomax 20/20, Promega, USA) and values were reported as mean ± SD.

Co-incubation of ED, FMN and AuNP1 in presence of EDC

Co-incubation of FMN and AuNP1 was carried out in the presence of ED and EDC. Four types of samples were prepared viz. 470 µL H_2O, 30 µL FMN; 410 µL AuNP1, 30 µL FMN, 60 µL H_2O; 410 µL AuNP1, 30 µL FMN, 30 µL EDC, 30 µL H_2O; and 410 µL AuNP1, 30 µL FMN, 30 µL EDC, 30 µL ED. The final concentrations of reagents in the solutions were 0.1 mM FMN, 10 µM EDC and 10 µM ED. The pre-assay mixture contained 300 µL phosphate buffer (pH: 7.0, 100 mM); 10 µL BLuc and NADH:FMN oxidoreductase; 50 µL 0.0025% tetradecanal solution and 50 µL 0.5 mM NADH. Bioluminescent reaction was initiated by addition of incubated samples (10 µL) to the pre-assay mixture. The luminescence measurements (n=3) were performed for 60 seconds using a luminometer (Glomax 20/20, Promega, USA) and values were reported as mean ± SD.

Results and discussion

Basic properties of synthesized gold nanoparticles

The synthesized gold nanoparticles (AuNP1-AuNP4) were faceted nanocrystals with localized surface plasmon resonance (LSPR) peak in the range of 520-535 nm (Fig. 1). The absorption maximum (Abs_{max}) and LSPR peak for AuNP1 was 0.99 and 520 nm respectively. AuNP2 had Abs_{max} of 1.24 and LSPR peak of 535 nm. Abs_{max} for AuNP3 and AuNP4 were 1.21 and 1.38 respectively and the LSPR peaks for AuNP3 and AuNP4 were 525 nm. The shift of the LSPR peak from blue towards red indicates that AuNP2, AuNP3 and AuNP4 exhibit a higher absorption in the red region than AuNP1 resulting in blue to purple colored AuNPs. Moreover, the shift tells about the size of AuNPs. The shift of LSPR peak towards the red region suggests increment in AuNPs size. Furthermore, higher visible absorbance of AuNPs indicates the higher concentration of nanoparticles. As the size increases, they tend to agglomerate and gradually settle to the bottom resulting in lower absorbance. However, it must be noted that a precise determination of AuNP concentration using this

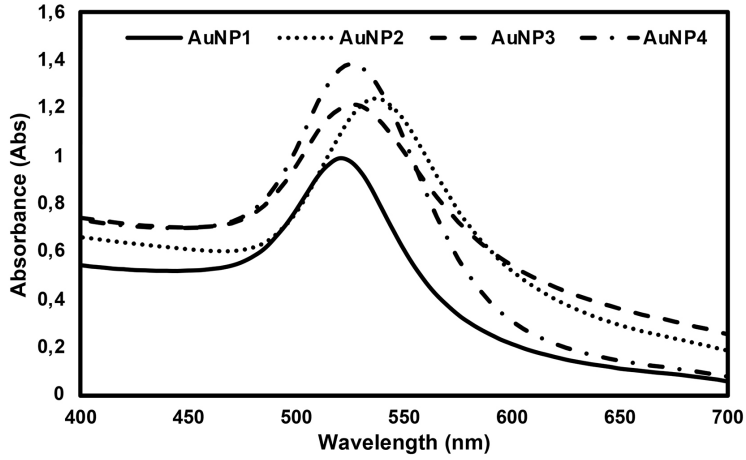

Fig. 1. Absorbance spectra of synthesized gold nanoparticles, AuNP1 (Abs_{max}: 0.99, λ_{max}: 520 nm); AuNP2 (Abs_{max}: 1.24, λ_{max}: 535 nm); AuNP3 (Abs_{max}: 1.21, λ_{max}: 525 nm); and AuNP4 (Abs_{max}: 1.38, λ_{max}: 525 nm)

method is rather difficult since the particles are not uniform in size. The yield of AuNPs followed the trend AuNP4>AuNP2>AuNP3>AuNP1.

Effect of EDC and ED on AuNPs stability

AuNPs aggregation can be visually observed. It takes less than a minute to observe color change from red to blue or purple in case the agglomeration of AuNPs occurs (Ranjan et al., 2018). The stability of AuNPs depends upon the capping agent. In citrate-capped AuNPs, the anionic shielding imparted by the negatively charged citrate ions prevents aggregation due to anionic repulsion. However, in case positively charged species come in the vicinity of AuNPs, the anionic shielding disrupts via charge neutralization, ultimately leading to aggregation. It was noted that molar strengths greater than 10 μM for both EDC and ED led to partial or complete aggregation depending upon the method used for synthesizing AuNP.

Effect of ED and EDC on luminescence response in AuNPs based FLuc assay

Luminescence enhancement up to 1.44-fold compared to the control (ATP) was observed predominantly in samples containing AuNP1 (Fig. 2, 1A, 1B, 1C). Ethylene diamine molecule has an amine group at its both ends and can form covalent bonds with the phosphate group of ATP and carboxylic group of AuNPs via coupling reactions. EDC is a water-soluble zero-length cross-linking agent which rapidly reacts with carboxylates or phosphates to form an active complex which allows coupling with amine-containing compounds (Hermanson, 2013). In the sample, containing ATP and AuNP1, a 1.32-fold luminescence enhancement was observed in post FLuc assay in comparison to control (Fig. 2, 1A). This increase in luminescence may be attributed to non-covalent weak interactions between positively charged adenine moieties of ATP and negatively charged citrate groups of AuNP1, which allowed optimal $d_{L\text{-}NP}$ to promote MEB. When EDC was introduced, a partial drop of 8.6% in luminescence was observed in comparison to the sample containing ATP and AuNP1 (Fig. 2, 1B). Moreover, partial aggregation was clearly visible in this case as the color change occurred. In a reaction with a carboxyl group EDC forms isourea as a by-product. Therefore, it is highly probable that the amine group of adenine present in ATP participates in the coupling reaction with

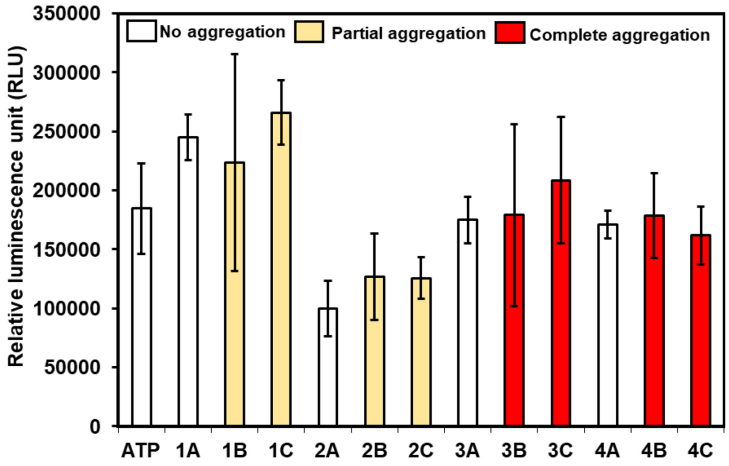

Fig. 2. Effect of ATP, gold nanoparticles (AuNP1 – AuNP4), carbodiimide (EDC), and ethylene diamine (ED) on luminescence response in firefly luciferase assay system. ATP: assay sample containing only ATP (control); 1A: ATP, AuNP1; 1B: ATP, AuNP1, EDC; 1C: ATP, AuNP1, EDC, ED; 2A: ATP, AuNP2; 2B: ATP, AuNP2, EDC; 2C: ATP, AuNP2, EDC, ED; 3A: ATP, AuNP3; 3B: ATP, AuNP3, EDC; 3C: ATP, AuNP3, EDC, ED; 4A: ATP, AuNP4; 4B: ATP, AuNP4, EDC; 4C: ATP, AuNP4, EDC, ED. Error bars show standard deviation for samples (n=3)

a carboxylic acid group of citrate-capped AuNP1. Covalent coupling between ATP and AuNP1 accompanied by the formation of a byproduct during the reaction might be responsible for this lowering of luminescence. Interestingly, when ED was introduced, the highest luminescence was observed (Fig. 2, 1C). The parametric conditions were favorable to observe MEB. However, no luminescence enhancement was observed when AuNP2, AuNP3 and AuNP4 were used for MEB. This result justifies the importance of the size, shape and nature of the nanomaterial used in MEB studies. Moreover, a complete aggregation was observed in the samples where ED and EDC were incubated with AuNP3 and AuNP4. The stability of AuNPs containing silver was found to be much lower than that of AuNP1. Hence further studies were carried out using AuNP1.

Effect of ED and EDC
on BLuc assay containing AuNP1

The combinations of FMN and AuNP1 and of FMN, AuNP1, ED and EDC did not enhance luminescence response when compared with the control (Fig. 3). In contrast, in the sample consisting of FMN, EDC and AuNP1 luminescence was 1.3-fold higher. FMN or riboflavin 5′-phosphate has a light sensitive isoalloxazine ring and a terminal phosphate group. When FMN was incubated with AuNP1, weak interactions were the least possible since the conjugated ring has no apparent positive charges to electrostatically interact with citrate ions of AuNP1. The negative charges on the terminal phosphate group would repel AuNP1 and thus rule out such possibilities. MEB was not observed in this case since it is a distance dependent phenomenon. When FMN, AuNP1, ED and EDC were incubated together, the formation of phosphoramidate bond between the amine group of ED and the phosphate group of FMN was possible. The other free amine group of ED might have interacted with a carboxyl group of AuNP1, but the $d_{L\text{-}NP}$ could have been unsuitable for obvious bioluminescence enhancement to occur. However, the systems with FMN, EDC and AuNP1 showed MEB, which suggests a close interaction of FMN with AuNP1 (at less than 10 nm).

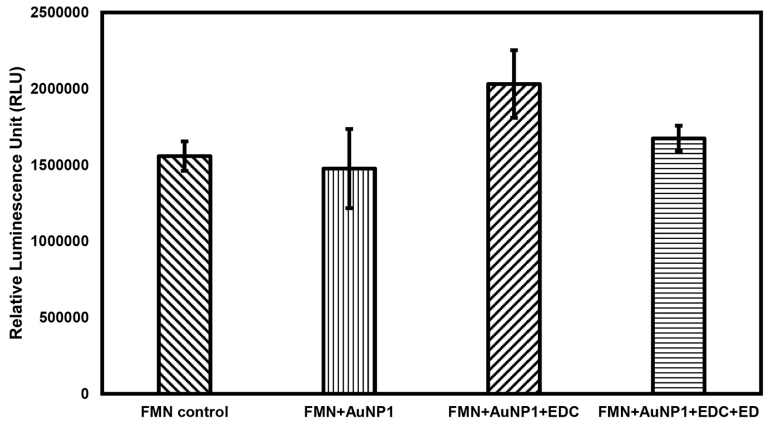

Fig. 3. Effect of flavin mononucleotide (FMN), gold nanoparticles (AuNP1), carbodiimide (EDC), and ethylene diamine (ED) on luminescence response in bacterial luciferase assay system. Error bars show standard deviation for samples (n = 3)

Conclusion

The study emphasizes the role of distance ($d_{L\text{-}NP}$) and the size, shape and nature of nanomaterials in observed MEB. Though the luminescence enhancement was below 1.5-fold, the role of ED and EDC in modulating luminescence is highlighted. ED as a linker was found to be a useful tool for understanding the distance-dependent MEB phenomenon. It is worth mentioning that when ED was used to covalently bind with ATP using EDC, luminescence enhancement was observed. On the contrary, when this method was applied using FMN instead of ATP, no substantial MEB was observed. In AuNP2, AuNP3 and AuNP4 silver ions played a negative role in MEB. It is highly probable that silver ions form a layer around these AuNPs and prevent covalent or ionic interactions between AuNPs, ED and bioluminescent substrates such as ATP or FMN. Luminescence enhancement approach seems to provide auspicious tools to design ultra-sensitive biosensors, therefore extensive research into this area is highly desirable.

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