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ARTICLE

Rapid and Sensitive Electrochemical Detection of MicroRNA by Gold Nanoparticle-catalyzed Silver Enhancement

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MicroRNAs(miRNAs) have played the vital role in the regulation of gene expression and have been considered as potential biomarker candidates for early cancer diagnosis. Rapid and sensitive detection of microRNAs is highly desired. Here we present a new method to rapidly and sensitively determine microRNAs based on the technology of gold nanoparticles catalyzed silver staining enhancement. The new method involves the sandwich hybridization of a capture probe immobilized on magnetic bead, a reporter probe assembled on gold nanoparticle and a miRNA target, catalytic silver precipitation by gold nanoparticles, magnetic collection of the enhanced sandwich complex, dissolution of the silver precipitation and stripping detection. The proposed method successfully fulfilled the microRNA-7a assay in less than 70 min and the detection limit was as low as 15 fM. The proposed biosensor may hold great promise in biological monitoring of microRNA.

Introduction

MicroRNAs (miRNAs), a major class of noncoding RNAs with 19-25 nucleotides in length,[1] play vital roles in the regulation of gene expression, such as growth, development, differentiation, reproduction, etc.[2-4] The unique characteristics of miRNAs, including small size, low abundance, and sequence homology, make them difficult to analyse.[5] Therefore, a highly selective and sensitive method for miRNA detection is greatly significant.

Due to its low cost, simple operation, good specificity and high sensitivity, the electrochemical techniques have been widely used in the quantitative analysis of miRNAs. Based upon the oxidation of guanine bases in hybridized miRNA strands, Schiavo[6] demonstrated a direct approach to miRNA quantitation without labeling. According to the changes in conductance after hybridization to miRNA, Fan et al.[7] successfully detected miRNAs in a direct way. By utilizing an amplification strategy based on Fe–Ru redox pair as reporter, Kelley[8] directly quantified miRNAs with a detection limit of 10 aM. To improve the sensitivity, several electrochemical biosensors for miRNAs detection have been fabricated based on the signal amplification such as enzymes,[9-12] inorganic nanoparticles,[13-15] metal compounds,[16, 17] and rolling circle amplification.[18-20]

Here we present a new method to rapidly and sensitively determine microRNAs based on the technology of gold nanoparticles catalyzed silver staining enhancement. The target miRNA was incubated with the mixture of the capture probe immobilized magnetic bead and the reporter probe assembled gold nanoparticle to form sandwich hybridization, followed by catalytic silver precipitation on the gold-nanoparticle tags. After a magnetic "collection" of the miRNA-linked sandwich complex, the silver aggregates was dissolved and used for stripping detection. Due to the multiple amplification, the new method showed excellent sensitivity comparable to or even better than the commonly used techniques.

Experimental

Reagents and materials

Chloroauric acid ($HAuCl_4$), trisodium citrate dihydrate and sodium lauryl sulfate(SDS) were purchased from Aladdin (Shanghai) Co., Ltd.. Hydroquinone, silver nitrate($AgNO_3$), citric acid, trisodium citrate, sodium thiosulfate pentahydrate and was purchased from Xilong Science and Technology Co.,Ltd.. Tris(2-carboxyethyl) phosphine hydrochloride(TCEP) and diethypyrocarbonate(DEPC) were purchased from Bio-Bioengineering (Shanghai) Co., Ltd.. Streptavidin-functionalized magnetic beads (MBs, 1 μm) was purchased from Bangs Lab (USA) Co.,Ltd. All chemical reagents were of analytical grade, and doubly Distilled water used was treated with DEPC. The oligonucleotides were synthesized from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of the miRNAs and ssDNAs were listed as following:

Table 1. Sequences of oligonucleotides

Name	Sequence (5′-3′)
miRNA-7a	UGAGGUAGUAGGUUGUAUAGUU
miRNA-7b	UGAGGUAGUAGGUUGUGUGGUU
miRNA-7e	UGAGGUAGGAGGUUGUAUAGU

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Capture Probe （CP）	CTACTACCTCATTTTTTTTTTTTTTTTTTT-biotin
Report Probe（RP）	SH-TTTTTTTTTTTTTTTTTTTAACTATACAAC

Note: The 5′ (underlined) and 3′ (dotted) terminal bases of miR-7a are complementary to the underlined sequence of the capture probe and the dotted sequence of the reporter probe, respectively. The underlined bases in miR-7b and miR-7e are the different bases as compared to miR-7a sequence.

Preparation of AuNPs and AuNP-RP

Gold nanoparticles were prepared according to the literature[21] with slight modifications. In brief, 100 mL of 0.01% chloroauric acid ($HAuCl_4$) solution was heated to reflux. Rapid addition of 3 mL of 1% sodiumcitrate was added to the boiling solution while stirring, which resulted in a color change from pale yellow to wine red. After the color change, the solution was kept boiling for an additional 15 min. Finally, the solution was stopped heating and cooling to room temperature. The resulted colloid was stored for use at 4°C in dark.

The AuNP-RP conjugates were prepared by adding thiolated RP to AuNPs solution (1 mL). 60 μL of 10 μM thiolated RP was first activated with phosphate buffer (pH 5.8) containing 10 mM TCEP for 1 h at room temperature. 1 mL of freshly prepared gold nanoparticles was then added and the mixture was shaken gently for 24 h. After that, the AuNP-RP conjugates were aged in 10 mM pH 5.8 PBS containing 0.1 M NaCl for another 24 h. The suspensions were removed by centrifugation at 12,000 rpm for 20 min. After washed three times, the obtained precipitates were dispersed in pH 7.4 PBS and stored for use at 4°C in the dark.

Preparation of MB-CP

50 μL of streptavidin-coated magnetic beads was washed three-times with 200 μL of imidazole buffer (0.1 M, pH 7.0) in a 2.5 mL centrifugal tube. The MBs were collected by magnetic separation and resuspended in 100 μL of 0.1 M PBS (pH 8.6). After adding 100 μL of 10 μM biotinylated CP solution, the mixture was incubated for 15 min with gentle mixing at room temperature. The resultant MB-CP conjugates were cleaned with 0.1 M imidazole buffer containing 0.5% SDS (pH 7.0) and RNase-free water for three times, respectively. Finally, the resultant conjugates collected were redispersed into 0.1 M Tris–HCl (pH 7.4, with 0.24 M NaCl) and stored in dark at 4°C for reserve.

Analysis procedure

The miRNAs samples were incubated with the mixture of 10 μL of the MB-CP solution and 10 μL of the AuNP-RP solution in the centrifugal tube at room temperature for 30 min. After washed twice with pH7.4 PBS, the resultant MB-conjugates were then treated with fresh silver enhancer solution (100 mg hydroquinone, 25 mg citric acid, 24 mg trisodium citrate, 30 mg sodium thiosulfate pentahydrate, 35 mg $AgNO_3$ and 10 mL deionized water) for 10 min in the dark, followed by full washing with pH 7.4 PBS.

200 μL of 0.5 M HNO_3 was added to the centrifugal tube and incubated at room temperature for 5 min to dissolve the silver clusters into silver(I) ions. The resulting solution was transferred into 3 mL electrolyte solution containing 0.1 M KNO_3/HNO_3 in a 10-mL electrochemical cell. The differential pulse stripping voltammetry (DPSV) was conducted to quantify the released silver(I) ions using an electrochemical analyzer (CHI660C, CH Instruments, Shanghai, China) with the following conditions: 5-min deposition at -0.5 V and the potential scan from +0.1 to +0.7 V at 100 mV/s scan rate. The conventional three-electrode system as used with a glassy carbon electrode as working electrode, a platinum wire as auxiliary electrode, and an Ag/AgCl as reference electrode. The peak current was recorded as the analytical signal. The working electrode was renewed by preconditioning at +1.0 V for 1 min between each measurement.

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Results and discussion

Principle of analysis

The procedure of the assay is described in Fig.1. The RP and CP were labeled with AuNP and MB to form Au–RP and MB–CP conjugates, respectively. After incubated with the testing samples, The RP and CP hybridized with the target miRNAs to forma sandwich-like complex, by which the AuNPs were brought onto the MBs. Excess unbound Au–RPs were removed by the washing with pH 7.4 PBS by a magnet. In the silver stainning process, silver ions were reduced by hydroquinone to silver metal on the surfaces of the AuNPs, in which the AuNPs promoted the silver deposition, and the deposited silver further catalyzed the silver reduction. The resulting Ag@Au-MB conjugates were then washed, collected and digested by HNO_3. The digested Ag^+ was determined by DPSV, providing the levels of the target miRNAs.

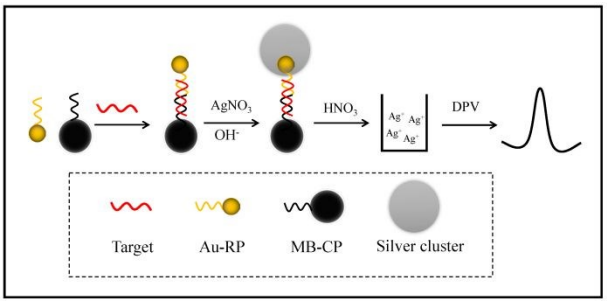

Fig. 1 Illustration of the electrochemical detection of miRNAs Based on the Silver Enhancement (not to scale).

Characterization of assay by TEM

The silver enhancement process was characterized by TEM in Fig.2. As shown in Fig.2A, the AuNP-RPs were well dispersed particles with an average diameter of ~16 nm. The MB-CPs showed an identifiable microspheres with a smooth surface and a diameter of ~1 μm (Fig.2B). when the target miRNAs of miR-7a (100 pM) was incubated with the mixture of MB-CPs and AuNP-RPs, the final collected MBs in Fig.2C showed small black dots(~16 nm) on the surface of MBs(~1 μm), resulting from the CP-MB/Target/Au-RP sandwich structure. It is observed from Fig.2D that the dots size was greatly increased after treatment in a silver developer solution. The obvious change in size indicated that silver deposition was successfully performed on the MB surface, in which the gold nanoparticles on MB served as nucleation sites and catalyzed the silver deposition.

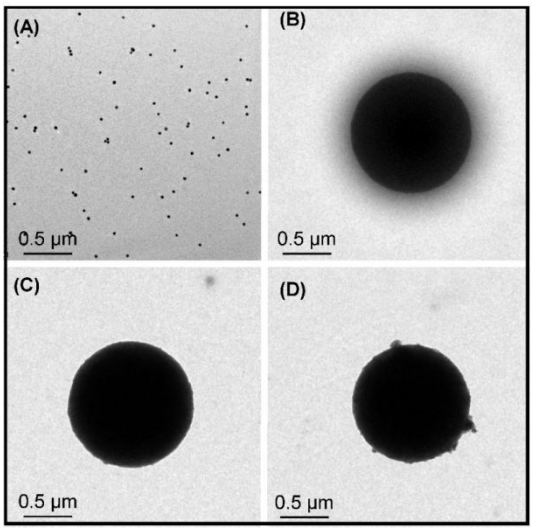

Fig.2 TEM images of AuNP-RPs (A), MB-CP(B), AuNP-RP/Target/CP-MB (C) and (C) after silver enhancement (D)

Optimization of experimental conditions

The proposed system is composed of hybridization, silver staining and electrochemical analysis. To obtain optimized results, the experimental parameters including the hybridization reaction time, silver staining time, deposition time and deposition potential were optimized in this work.

The hybridization time was closely related to hybridization efficiency due to a steric hindrance arising from the bead and the AuNPs. Thus, the dependence of DPSV signal on the hybridization time was investigated from 5 min to 60 min. As presented in Fig.3A, the signal increased with the increase of incubation time and then reached a plateau when it exceeded 40 min. Therefore, 40 min was chosen as the optimum hybridization time.

Silver staining time was the crucial factor, which directly affected the amplification efficiency. Fig.3B showed the effect of the time from 3 to 18 min on the peak current. The signal-to-background ratio increased significantly as the staining time increased up to 12 min. After 12 min, However, the ratio decreased with the time, which was due to the increased background signal resulted from the nonspecific stainning. In order to obtain higher performance, 12 min was chosen as the optimum staining time.

Deposition time and deposition potential had important influence on the sensitivity of the method. The effect of the deposition potential from −0.80 to −0.30 V was studied. In Fig.3C, the response current increased greatly and reached the maximum value at −0.50 V. The influence of the deposition time from 150 to 450 s upon the analysis was examined under the deposition potential of −0.50 V in Fig.3D. The current increased along with the time up to 300 s, and leveled off after that. Therefore, stripping analysis was carried out under deposition potential of −0.50 V with the deposition time of 300 s.

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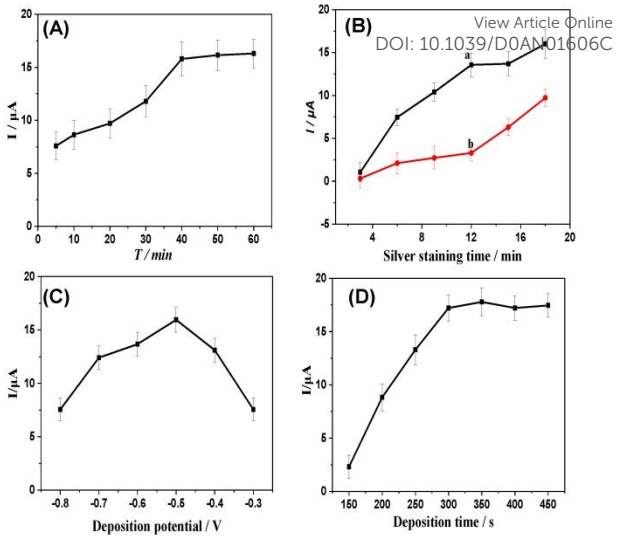

Fig.3 Optimizations of experimental conditions: hybridization time (A), silver staining time (B), deposition time (C) and deposition potential (D).

Sensitivity of the assay

After optimizations of the conditions, the proposed method was challenged by the target miR-7a with different concentrations (Fig.4). The peak current increased along with increased miR-7a concentration. The current was linearly correlated with the target concentration from 50 fM to 250 pM. The linear equation was I (μA)= 44.093 + 3.0769 log C (C was the concentration of miR-7a, M), with a linear correlation coefficient of 0.991, and the detection limit(LOD, at an S/N ratio of 3) was 15 fM. It is comparable to or even better than the commonly used techniques, such as northern blotting (LOD = 1 nM),[22] electrochemical assay (LOD = 2 pM),[23] fluorometric assay (LOD = 25 fM),[24] SPR imaging (LOD = 10 pM),[25] and fluorescence correlation spectroscopy (LOD = 500 fM).[26] The high sensitivity was due to the dual amplification based on gold nanoparticle-catalyzed silver enhancement, in which AuNPs with large surface areas could serve as nucleation sites for silver deposition and catalyze the deposition . In addition, the deposited silver further induced the silver reduction, resulted in a large amount of silver clusters.

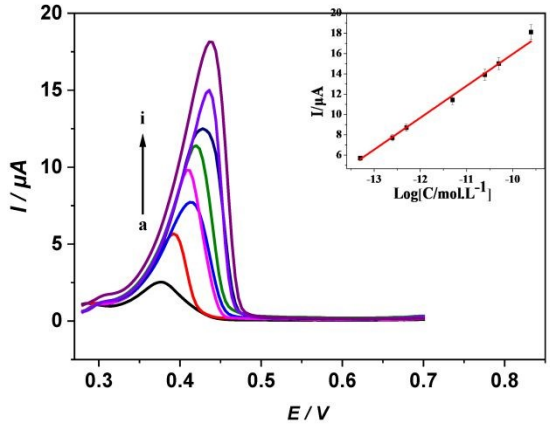

Fig.4 DPSV responses of the biosensor to miR-7a with different concentrations (form a to i: 0, 0.05, 0.25, 0.5, 5, 25, 50, 250 pM) in

0.1 M KNO_3/HNO_3. The inset displays the linear relationship between the peak current and the logarithm of the target miRNA-7a concentration.

Specificity of the method

It is a great challenge to distinguish between miRNAs due to its high sequence homology. To evaluate the specificity, the proposed method was challenged by single-base mismatched (miR-7e) and two-base mismatched (miR-7b) miRNAs. As shown in Fig.5, the signal intensities for miR-7e and miR-7b at the concentration of 1 nM were close to that of the blank. However, in the presence of target miR-7a (100 pM), the intensity had a remarkable increase, demonstrating that this method was able to discriminate miRNAs that differ in sequence.

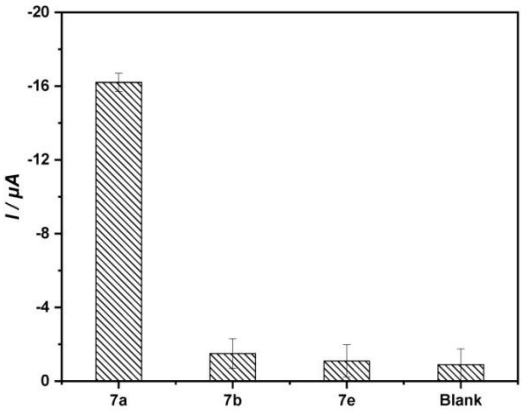

Fig.5 The responses of the biosensor to miR-7a (100 pM), miR-7b (1 nM), miR-7e (1 nM) and blank. Error bars are standard deviations across four repetitive experiments.

Analytical application

To investigate the performance of the method, healthy human sera, which were provided by the affiliated hospital of guilin university of technology, were analyzed to determine their miR-7a contents. The samples(1.0 mL) were prepared by adding 20 mL perchloricacid (HClO4, 20% v/v) to precipitate proteins. After centrifugation at 1000 rpm for 20 min, the obtained solution was diluted five times with water and transferred into the electrochemical cell to be analyzed. The initial concentrations of miR-7a in the samples were undetectable. According to the standard addition method, three different concentrations (1 pM, 5 pM and 10 pM) of chemically synthesized miR-7a were spiked into the samples. As shown in Table 2, the recovery values were in the range of 97.2% to 102.0% and the RSDs were 1.6–2.3%. These results suggested that the established miRNA biosensor could be applied to detect the miRNA in real samples with satisfactory results.

Table 2 Assay results of spiking miR-7a into human plasma by the electrochemical biosensor

Samples	Spiked(pM)	Detected(pM)	Recovery(%)	RSD(%)
Sample-1	0	Undetectable		
Sample-2	1	1.02	102.0	2.3
Sample-3	5	4.86	97.2	1.8
Sample-4	10	100.4	100.4	1.6

Conclusions

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A rapid and sensitive method was developed to detect miRNAs based on silver enhancement. The new method showed excellent sensitivity with the detection limit of 15 fM. The high sensitivity was due to the amplification based on gold nanoparticle-catalyzed silver enhancement, in which AuNPs with large surface could serve as nucleation sites for silver deposition and catalyze the deposition and the deposited silver further induced the silver reduction. The proposed method was successfully used to determine miR-7a in less than 70 min. The proposed biosensor may hold great promise in biological monitoring of microRNA.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Notes and references

1. V. Ambros, *Cell*, 2001, **107**, 823-826.
2. G. Hutvagner and P. D. Zamore, *Science*, 2002, **297**, 2056-2060.
3. Z. Mourelatos, J. Dostie, S. Paushkin, A. Sharma, B. Charroux, L. Abel, J. Rappsilber, M. Mann and G. Dreyfuss, *Genes & Development*, 2002, **16**, 720-728.
4. V. Ambros, *Cell*, 2003, **113**, 673-676.
5. H. T. Allawi, *Rna*, 2004, **10**, 1153-1161.
6. E. A. Lusi, M. Passamano, P. Guarascio, A. Scarpa and L. Schiavo, *Analytical chemistry*, 2009, **81**, 2819-2822.
7. Y. Fan, X. Chen, A. D. Trigg, C.-h. Tung, J. Kong and Z. Gao, *Journal of the American Chemical Society*, 2007, **129**, 5437-5443.
8. H. Yang, A. Hui, G. Pampalakis, L. Soleymani, F.-F. Liu, E. H. Sargent and S. O. Kelley, *Angewandte Chemie International Edition*, 2009, **48**, 8461-8464.
9. Z. Gao, *Analyst*, 2012, **137**, 1674-1679.
10. T. Kilic, S. N. Topkaya, D. O. Ariksoysal, M. Ozsoz, P. Ballar, Y. Erac and O. Gozen, *Biosensors & Bioelectronics*, 2012, **38**, 195-201.
11. Y. Zhou, M. Wang, X. Meng, H. Yin and S. Ai, *Rsc Advances*, 2012, **2**, 7140-7145.
12. S. Wen, Y. Su, C. Dai, J. Jia, G.-C. Fan, L.-P. Jiang, R.-B. Song and J.-J. Zhu, *Analytical Chemistry*, 2019, **91**, 12298-12306.
13. Z. Gao and Z. Yang, *Analytical chemistry*, 2006, **78**, 1470-1477.

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DOI: 10.1039/D0AN01606C

14. Y. Peng and Z. Gao, *Analytical chemistry*, 2011, **83**, 820-827.
15. Q. Liu, C. Ma, X.-P. Liu, Y.-P. Wei, C.-J. Mao and J.-J. Zhu, *Biosensors & Bioelectronics*, 2017, **92**, 273-279.
16. M. Labib, N. Khan, S. M. Ghobadloo, J. Cheng, J. P. Pezacki and M. V. Berezovski, *Journal of the American Chemical Society*, 2013, **135**, 3027-3038.
17. S. Xue, Q. Li, L. Wang, W. You, J. Zhang and R. Che, *Analytical Chemistry*, 2019, **91**, 2659-2666.
18. A. Chen, S. Ma, Y. Zhuo, Y. Chai and R. Yuan, *Analytical Chemistry*, 2016, **88**, 3203-3210.
19. 19. J. Yang, M. Tang, W. Diao, W. Cheng, Y. Zhang and Y. Yan, *Microchimica Acta*, 2016, **183**, 3061-3067.
20. 20. Q. Li, F. Zeng, N. Lyu and J. Liang, *Analyst*, 2018, **143**, 2304-2309.
21. 21. K. C. Grabar, R. G. Freeman, M. B. Hommer and M. J. Natan, *Analytical chemistry*, 1995, **67**, 735-743.
22. 22. P. T. Nelson, D. A. Baldwin, L. M. Scearce, J. C. Oberholtzer, J. W. Tobias and Z. Mourelatos, *Nature methods*, 2004, **1**, 155-161.
23. 23. H. Hatakeyama, H. Cheng, P. Wirth, A. Counsell, S. R. Marcrom, C. B. Wood, P. R. Pohlmann, J. Gilbert, B. Murphy, W. G. Yarbrough, D. L. Wheeler, P. M. Harari, Y. Guo, Y. Shyr, R. J. Slebos and C. H. Chung, *Plos One*, 2010, **5**.
24. 24. A. Singer, M. Wanunu, W. Morrison, H. Kuhn, M. Frank-Kamenetskii and A. Meller, *Nano Letters*, 2010, **10**, 738-742.
25. 25. J. M. Lee, H. Cho and Y. Jung, *Angewandte Chemie International Edition*, 2010, **49**, 8662-8665.
26. L. A. Neely, S. Patel, J. Garver, M. Gallo, M. Hackett, S. McLaughlin, M. Nadel, J. Harris, S. Gullans and J. Rooke, *Nature methods*, 2006, **3**, 41-46.

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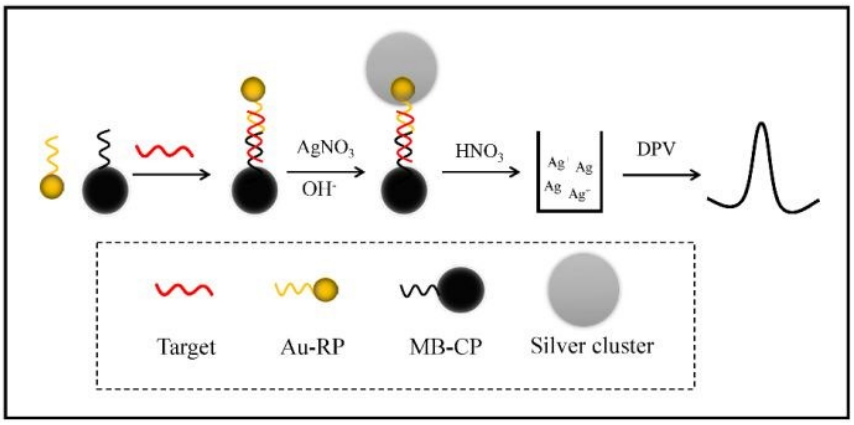

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