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Nanoparticle-GFP “chemical nose” sensor for cancer cells identification

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Summary

Nanoparticle-based sensor arrays have been used to distinguish a wide range of bio-related molecules through pattern recognition. This “chemical nose” approach uses nanoparticles as receptors to *selectively* identify the analytes, while a transducer reports the binding through a readable signal (fluorescence). Here we describe a procedure that uses functionalized gold nanoparticle as receptors and Green Fluorescent Protein (GFP) as the transducer to identify and differentiate cell state (normal, cancerous, and metastatic), an important tool in early diagnosis and treatment of tumors.

Keywords

Sensor; chemical nose; gold nanoparticle; GFP; fluorescence; LDA

1. Introduction

Antibody-based sensing techniques are an important tool in the early detection of cancer (1). These techniques employ *specific* recognition to identify the analytes, targeting different biomarkers of each cell state (2). However this approach also limits the applicability of this method due to constraints in the availability of specific markers (3). As an alternative to this methodology, the “chemical nose” approach utilizes multiple *selective* receptors that generate a unique response pattern for each analyte, allowing its classification (4). The identification is achieved by taking advantage of differential interactions between the analytes and the receptors. In the sensor approach described here, gold nanoparticles (AuNPs) are used as the receptors, controlling the nature of the interaction by tuning the chemical properties at the nanoparticle surface (5). AuNP-analyte interactions are then transduced by a fluorescent probe (GFP), initially quenched when bound to the AuNP, and then displaced from the AuNP surface upon the addition of the analyte, with concomitant restoration of fluorescence. This strategy has been successfully applied to identify and differentiate a variety of bioconstructs, from proteins (6) to bacteria (7) and cancer cells (8). Here we report the procedure to apply this methodology to the identification of cancer cells, independent of their origin (isogenic), and differentiating among a diversity of cell states (normal, cancerous and metastatic). This strategy allows a versatile identification of cellular

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signatures in early stages of cancer, a major hurdle in cancer therapy, without previous knowledge of specific receptors or ligands.

2. Materials

1. Phosphate buffer (PB) solution: 5 mM phosphate, pH 7.4. Mix 3.87 mL of 1.0 M Na_2HPO_4 with 1.13 mL of 1.0 M NaH_2PO_4 solution. Complete to a final volume of 1.0 L using Type I ultrapure water (*see Note 1*). Adjust pH if necessary and store at room temperature (25°C).
2. Functionalized gold nanoparticles solution: 3 cationic and 1 neutral gold nanoparticles (**Fig. 1**) are synthesized by place exchange reaction (9), from pentanethiol-capped 2nm diameter gold core nanoparticles, and using the desired ligands (synthesized as shown in (10)). Prepare a 40 μM stock solution of each nanoparticle using Type I ultrapure water and store at -4°C (*see Note 2*).
3. Green fluorescent protein solution: GFP is expressed according to the reported procedure (11). Prepare a GFP stock solution of 250 μM in 5 mM PB, calculating the protein concentration by its absorbance at 488nm ($\epsilon_{488} = 5.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (*see Note 3*).
4. Cell suspension: Grow cancer, metastatic and normal state cells (analytes) according to the provider procedures in T75/T25 flasks. Remove the media, wash the cells with DBPS buffer, add 2mL of Trypsin EDTA 1X (per container), and collect the cells in serum-containing media. Centrifuge to obtain the cells pellet and suspend them in DMEM non-serum media. Count the cells and dilute the suspension with DMEM to have a final concentration of 200 cells/ μL (*see Note 4*).
5. 96 Well 300 μL Black Polystyrene plates with dark flat bottom.
6. 50mL PVC pipette basins, 50mL PP Falcon tubes and 7mL glass vials.

3. Methods

All procedures are done at room temperature.

¹The 1.0 M solution of NaH_2PO_4 has to be fresh each time that the buffer is prepared to avoid later crystallization problems of this solution that can alter the final concentration.

²After place exchange reaction, nanoparticles are purified by dialysis using a 10,000 MWCO tubing membrane. The nanoparticles are then lyophilized, dispersed in Type I ultrapure water, and passed through a 0.22 μm membrane filter. TEM, MS, NMR and DLS techniques are used to control the quality of the nanoparticles according to literature (10). The final concentration was calculated following the reported extinction coefficients methodology for 2nm diameter gold core nanoparticles (12).

³Continuous freezing cycles of GFP can denature the protein, affecting the reproducibility. To avoid this problem, prepare the stock solution and divide it in different containers, store them at -78°C in dark (aluminum foil), and take out one container each time that the solution is needed. No glycerol or DTT is used given the stability of GFP in PB at low concentrations.

⁴If not recommendations are given, grow Human and Mouse type cells in Dulbecco's Modified Eagle's Medium (DMEM, 4.0 g/L glucose) supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) in T75 or T25 flasks, and under humidified atmosphere of 5% CO_2 at 37°C . The cells should be maintained in the mentioned conditions and subcultured once every four days.

3.1. Determination of optimal sensor conditions

1. Prepare 40 mL of 150 nM GFP solution in a Falcon tube using 5 mM PB, starting from the initial GFP stock solution (*see Note 5*).
2. In a 7 mL glass vial, pour 10 μ L of one of the nanoparticles stock solutions. Complete the solution to 2 mL using the 150 nM GFP solution. Mix gently and allow this AuNP-GFP complex solution to reach equilibrium for 15 min (*see Note 6*).
3. During the equilibration time, add sequentially decreasing values of the 150 nM GFP solution into a 96 well plate, according to the scheme shown in **Fig. 2**.
4. After the 15 minutes, add sequentially increasing values of the AuNP-GFP complex solution to each well according to the scheme shown in **Fig. 2**, to complete a total volume of 200 μ L per well. Each nanoparticle has to be titrated by triplicate (*see Note 7*).
5. Mix gently with the pipette tip to avoid bubbles and leave the system reach equilibrium for another 15 min.
6. After the final equilibration time, take fluorescent intensity measurements at 510 nm, using an excitation wavelength of 475 nm.
7. Plot the normalized fluorescence intensity against the nanoparticle concentration in each well (*see Note 8*).
8. Select the ideal AuNP/GFP ratio for sensing purposes, as shown in **Fig. 3**. This is the ratio to be used in the **method 3.2** (*see Note 9*).
9. Repeat the same procedure using the four different nanoparticles. The AuNP/GFP optimal ratio may differ among all nanoparticles (*see Note 10*).

3.2. Sensing procedure

1. Prepare 40 mL of 150 nM GFP solution in Falcon tube using 5 mM PB and starting from the initial GFP stock solution (*see Note 5*).

⁵Maintain the 150 nM GFP solution in an ice bath and cover the Falcon tube with aluminum foil while performing the experiments to avoid photobleaching and denaturation. Homogenize the solution by mixing gently.

⁶During the equilibrium times, the system should be maintained in dark conditions (covering the plate/vial with aluminum foil).

⁷Given that each nanoparticle can be titrated (with its three replicates) in 48 wells, 2 different nanoparticles can be analyzed per plate, optimizing the time performance.

⁸The normalized fluorescence is obtained by:

$$\| \mathbf{I} \| = (I_c / I_n) / (I_c^o / I_n^o)$$

Where I_c is the intensity of a given point of a cationic nanoparticle, I_n is the intensity of the same point of the neutral (control) nanoparticle, and I_c^o and I_n^o are the measurements in the absence of nanoparticles in the solution (first point). This is done to eliminate the effect of the gold core natural absorption.

⁹Choose a point in the curve at the bottom of the plot, after the principal quenching (initial slope) but before reaching the horizontal tendency. Points in this zone have the biggest I after the addition of the analyte due to its sensitive nature in the competitive binding (13). The control nanoparticle does not have a titration point, in this case, use a concentration similar to the cationic nanoparticles.

¹⁰The total time used to analyze each nanoparticle should be consistent in all the cases to avoid reproducibility problems. Use the same amount of time when preparing as well as mixing the solutions for each one of the nanoparticles.

2. In a 7 mL vial, pour the amount of the nanoparticle stock solution necessary to reach the optimal AuNP/GFP ratio (150 nM of GFP), completing to 2 mL using the 150 nM GFP solution. Mix gently and wait during 15 min. Repeat this step for the other nanoparticles (*see Notes 6 and 10*).
3. After the equilibrium time, add 200 μ L of each AuNP/GFP solution in the 96 wells plate according to the scheme in **Fig. 4** (*see Note 11*). Six replicates are done for each specific nanoparticle.
4. Record the fluorescent intensity at 510 nm, using an excitation wavelength of 475 nm. This is the initial intensity (I_i).
5. Add 25 μ L of the cell suspension (5000 cells) in each well (*see Note 12*). Mix with the pipette tip gently and leave 30 min for incubation time (*see Note 6*).
6. After the incubation time, record the fluorescent intensity at 510 nm, using an excitation wavelength of 475 nm. This is the final intensity (I_f).
7. Calculate the $\text{Log}(I_f/I_i)$ for each point, generating the average and the standard deviation with the six replicates, to build up the fingerprint of each analyte (*see Note 13*).
8. Using Linear Discriminant Analysis software (like Systat 11), use all $\text{Log}(I_f/I_i)$ values (analytes x replicates x nanoparticles) as an input to generate the canonical score plots and the Jackknife classification percentage for cell identification (*see Notes 14 and 15*).

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¹¹We find that the minimum amount of cationic nanoparticles as predictors to have a good percentage of identification (>90%) is three, featuring functional groups for h-bond, π -stacking and hydrophobic interactions (14). However more nanoparticles can be used to increase identification accuracy.

¹²As deduced from **Fig. 4.**, four different cells can be run simultaneously in the same plate, optimizing the analysis process.

¹³We find that six is a reasonable number of replicates to have good representation in the canonical graphs with a 95% of confidence (no overlapping).

¹⁴LDA generates canonical factors to minimize the training matrix. The best two canonical factors are the ones used to generate the 2D score plots.

¹⁵In the case of an unknown test sample, follow **method 3.2** with the unidentified cell line. Using Mahalanobis distance analysis, compare the results with the previously generated matrix for proper identification. This analysis is done using Systat as described above.

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Fig. 1. Chemical structure of the gold nanoparticles, featuring h-bond, π -stacking and hydrophobic groups at the surface.

Fig. 2. Sequential volumes of the GFP and AuNP-GFP solutions to be used in the 96 wells plate for titration purposes.

Fig. 3. Cationic-AuNP titration curve featuring the position of the optimal AuNP/GFP ratio to use in the sensing procedure.

Fig. 4. Input scheme to use the different AuNP-GFP complexes against one cell analyte. The output of this experiment generates the fingerprint for the given analyte.