Tween-modified suspension array for sensitive biomolecular detection

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Suspension arrays are attracting increasing interest for detecting and quantifying proteins, nucleic acids and other biomolecules. Among various suspension arrays, silica-based suspension arrays which benefit from low fluorescence background and material stability have been widely used. Silica-based suspension arrays are often manufactured with the popular aldehyde-aminosilane chemistry for the attachment of a variety of biomolecules. One drawback of this immobilization strategies is the relatively high array particles lost efficiency when washed by centrifugation. Due to this shortcoming, it is low reproductivity and limited in multiplex assay. Herein we report a novel method to fabricate Tween-coated suspension silica particles, which could achieve good reproductivity and the low limit of detection. Tween surfactants, each containing hydrophilic ethylene glycol head groups and a hydrophobic alkyl tail, prevent silica particles from being adsorbed onto the centrifuge tube wall and make beads resuspended well. Also, Tween with low fluorescence background could reduce non-specific protein adsorption. Also Tween surfactants are economic and facile agent. In this study, we demonstrate the preparation of the protein array to substantiate the applicability of our approach. Under the optimized experiment conditions, the limit of detection of our Tween-modified particles is as low as 50 pg/mL, which is sufficiently low for the current methods. We believe that the proposed method could provide a perspective on the improvement of self-encoded silica particle arrays.

Tween-20 surfactant, surface modification, suspension array, non-specific adsorption

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Recent advances in chemical biology and bioengineering have led to the development of biosensors and biomolecules analysis [1]. In particular, microarrays provide an efficient way for the multiplexed analysis. These technologies have substantially influenced many areas, such as genetic analysis, clinical diagnosis. There are two broad classes of microarrays: planar arrays and suspension arrays. Suspension arrays, which use encoded nano or micro particles in solution as elements, are attracting increasing interest for detection and multiplex analysis of proteins, nucleic acid and

other biomolecules [2–4]. Due to faster reaction kinetics and higher sample throughput, protein suspension arrays have become increasingly important tools for protein interaction studies and diagnostics [5].

Suspension arrays usually involve Luminex beads (Luminex's xMAP Technology) [6], which are encoded by spectral. Although this method has already been commercial, these fluorescence-encoded bead arrays suffer from several disadvantages, including the limited barcodes achievable, the higher costs for manufacture and detection, and the potential spectral overlap of fluorescent signals between encoding and analyte detection in multiplexed assays [7]. Considering those drawbacks, graphically encoded particles

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have been developed. Some typical cases include Illumina's VeraCode technology, dot-coded polymer particles [8], color-barcode magnetic microarray [9] and shape-encoded silica nanotubes [10]. By virtue of low intrinsic fluorescence background, ease of photolithographic technology, and a broad range of available surface chemistries for immobilization, SiO₂ particles are widely used for suspension arrays.

Non-specific protein adsorption is the main source of noise in immunoassays and has become the major obstacle in developing more sensitive and high throughput immunoassays. In order to get a lower limit of detection, many methods have been developed, including three-dimensional (3D) matrixes modification, such as Noble PA group and Bavykin S group developed polyacrylamide for surface modification [11,12], and Gershwin ME group and Dufva M group used agarose for surface coated and polymer [13,14], which could increase the efficiency of biomolecules immobilization. And some groups chose signal amplification technology [15–19], or used more sensitive signal detection and collection apparatus for signal collection. In our study, we developed a method with Tween coated silica particles that makes the high productivity of the particles. Although this method only contains two-dimensional (2D) matrixes modification and uses normal fluorescence microscope, we could achieve even lower limit-of-detection. Also, the chemical modification of Tween needs the mild reaction condition, and the detection does not need the special apparatus, so we decreased the cost for fabricating the suspension array and simplified the experiment procedure. We hope that this method could be widely applied into self-encoded SiO₂-based suspension arrays for biological and medical analysis.

1 Materials and methods

1.1 Chemical and reagents

Silica beads (4.5 μm) were obtained from Bangs Laboratories, Inc. 3-(2-Aminoethylamino)propyldimethoxymethylsilane, 3-glycidioxypropyltrimethoxysilane, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 4-(Dimethylamino)pyridine (DMAP), N-hydroxysuccinimide (NHS) and Tween-20 were obtained from Sigma-Aldrich. DyLightTM 488-conjugated goat anti-rabbit IgG (DL488 anti-rabbit IgG) was purchased from Jackson Immuno Research Labs. Other proteins were obtained from ZhongShan Golden Bridge Biotechnology Co. Ltd. All chemicals and solvents were of analytical grade and deionized water was used throughout the experiment.

1.2 Synthesis of (3-carboxy-1-oxopropyl)polyethylene glycol sorbitan monolaurate (carboxyderived-Tween)

Tween-20 (4 mmol), succinic anhydride (6 mmol) and DMAP (0.2 mmol) were mixed well in 20 mL dry acetonitrile,

and was refluxed overnight with continuous stirring. Then the solution was cooled to room temperature. The solvent was evaporated under reduced pressure [20]. The oil solvent was stored at room temperature.

1.3 Preparation of carboxyl derived-Tween, succinic anhydride, and succinic acid modified epoxy-silanized beads

Silica beads were modified as described previously with the following changes. The beads (~10⁶) were washed with 95% ethanol (v/v) prior to derivatization with the silane reagent. Silica beads were suspended in 500 μL of 10% 3-glycidioxypropyltrimethoxysilane solution in 95% ethanol for 1 h at room temperature with gentle shaking [21]. Following silanization, epoxy-silanized beads were rinsed thoroughly with ethanol followed by N,N-dimethylformamide (DMF). Finally the beads were suspended in 100 μL DMF. Subsequent derivatization of the epoxy-silane with Tween spacer was performed as reported previously, the epoxy beads were reacted with 20% carboxyl derived-Tween, 20% succinic anhydride, and 20% succinic acid in DMF, respectively, with a catalytic amount concentrated of sulfuric acid (H₂SO₄) with continuous stirring overnight at 50°C [22]. Then the beads were rinsed with DMF and water.

1.4 Preparation of succinic anhydride and succinic acid modified amino-silanized beads

The 95% ethanol washed silica beads were treated with 500 μL of 5% 3-(2-aminoethylamino)propyl dimethoxymethylsilane solution in 95% ethanol for 0.5 h at room temperature with gentle shaking [21]. After silanization, amino-silanized beads were rinsed thoroughly with ethanol and then washed by DMF. Amino-silanized beads were suspended in DMF and reacted with 10% succinic anhydride in DMF solution under N₂ gas for 6 h with continuous shaking [23]. Amino beads were reacted with 10% succinic anhydride with addition of a catalytic amount of 4-(dimethylamino)pyridine (DMAP) in DMF solution under N₂ gas for 4 h with continuous shaking. Those beads were washed by water.

1.5 Immobilization of proteins on modified beads

Fluorescence-labeled protein (DL488 anti-rabbit IgG) was directly immobilized on the surfaces of different modified beads for signal detection. After thorough water rinsing, the modified carboxylated beads were activated with the mixed solution (50 mg/mL N-hydroxysuccinimide (NHS) and 100 mg/mL EDC in 100 mmol/L pH 5.0 MES buffer) for 0.5 h at room temperature with continuous shaking. Then, the activated beads were washed and resuspended in 10 mmol/L pH 5.0 acetate buffer to react with 100 μL 7.5 μg/mL

DL488 anti-rabbit IgG for 4 h in the dark with gentle shaking. Finally, the beads were washed with PBST (pH 7.4 containing 0.1% Tween-20) for signal detection.

In order to prepare protein array, goat anti-rabbit IgG (40 $\mu\text{g/mL}$) was added to activated carboxyl-Tween coated beads with continuous shaking for 4 h. After washing, the beads were resuspended in 50 mmol/L pH 7.4 Tris buffer for 0.5 h at room temperature to quench the unreacted activated carboxylic acid groups, followed by 1% BSA in PBS for 0.5 h. Then the 5000 beads were reacted with 50 $\mu\text{g/mL}$ 100 ng/mL rabbit IgG. After washed by PBST buffer, the beads were incubated with 7.5 $\mu\text{g/mL}$ DL488 anti-rabbit IgG for 2 h in the dark, and then rinsed by PBST buffer.

As a typical experiment for protein detection, sandwich immunoassay is illustrated in Figure 1. We immobilized goat anti-rabbit IgG (40 $\mu\text{g/mL}$) on carboxyl-Tween coated beads. Then, the beads were incubated with 2 $\mu\text{g/mL}$ rabbit IgG, human IgG, goat Ig and BSA for 2 h, respectively. After washed by PBST buffer, the beads were incubated with 7.5 $\mu\text{g/mL}$ DL488 anti-rabbit IgG for 2 h in the dark, rinsed and imaged to determine fluorescent intensity.

2 Discussion

Reactions about beads were performed in microcentrifuge tubes at room temperature with continuous shaking by the Vortex-Genie 2 mixer. Beads were rinsed by centrifugation to remove the supernatant at 9000 r/min. All fluorescence images were performed with Nikon Eclipse Ti inverted research microscope. A volume of 1 μL of beads reaction solution was dropped onto a hydrophobic glass slide. Then we performed the image before the droplet was dried, and all the optical parameters were kept constant in related measurement. We used other modification, and we found that the beads would be adsorbed on the centrifuge tube wall. Also, the beads formatted the trailing. When removing the supernatant, beads would be taken away, it is limited for multiplex assays when using few beads. And Tween-20 modified

beads have not appeared the trailing, and we thought that Tween has the potential advantage for modification.

2.1 Identify the conjugation of Tween onto the beads

As shown in Figure 2, the high fluorescent signal from carboxyl derived-Tween coated epoxy beads, and the low fluorescent signal from succinic anhydride and succinic acid coated epoxy beads, illustrated that carboxyl derived-Tween was conjugated onto epoxy beads, and succinic anhydride and succinic acid were not modified to the surface of epoxy beads. So in our protocol, no effort is put into the purification of the synthesized carboxyl derived-Tween oil mixture and dialysis of carboxyl derived-Tween prior to use.

2.2 Optimization of carboxyl-Tween coating on epoxy beads

Here, we put emphasis on the dependence of carboxyl-Tween coating on the temperature of the reaction, carboxyl-Tween concentration and the mole ratio of Tween to succinic anhydride. Under the constant condition that the mole ratio of Tween and succinic anhydride is 1:2 and the concentration of carboxyl derived-Tween is 20% (m/v), epoxy beads reacted with carboxyl derived-Tween at room temperature, 50°C, 75°C, respectively. Figure 3 shows that 50°C is the best temperature for the reaction of epoxy of beads and hydroxyl of Tween in our experiment.

When synthesizing carboxyl derived-Tween, we used the mole ratio of Tween-20 to succinic anhydride 1:1, 1:1.5, 1:2, respectively. Under the condition of 50°C and 20% (m/v) carboxyl derived-Tween, we modified epoxy beads with the mole ratio of Tween-20 and succinic anhydride 1:1, 1:1.5, 1:2, respectively. And we immobilized DL488 anti-rabbit IgG on those three carboxyl derived-Tween coated epoxy beads, respectively. Figure 2 demonstrates that the mole ratio of Tween-20 to succinic anhydride 1:2 and 1:1.5 has the same immobilization capacity and uniform signal. The mole ratio of Tween-20 and succinic anhydride 1:2,

Figure 1 Schematic representation of protein arrays using carboxyl derived-Tween coated particles.

Figure 2 Detection of immobilized DL488 anti-rabbit IgG on succinic anhydride-coated beads, succinic acid-coated beads and Tween-coated beads, respectively.

Figure 3 Detection of immobilized DL488 anti-rabbit IgG on Tween-coated beads under different temperatures, different ratios of succinic anhydride and Tween, different carboxyl-Tween concentrations, respectively.

synthesized carboxyl derived-Tween oil would adsorb crystal after more than one month. So we chose the ratio of Tween-20 and succinic anhydride 1:1.5 for our following experiment.

Also we sought to determine the optimal concentration of the carboxyl derived-Tween that yielded the highest signal from immobilized DL488 anti-rabbit IgG on this modified beads. Under the following optimized condition, we coated the epoxy beads with the concentration of carboxyl derived-Tween 5%, 10%, 20%, respectively. As shown in Figure 3, the fluorescence signal is no distinction between the concentration of carboxyl derived-Tween 10% and 20%. We chose 10% concentration as our following experiment condition, due to its higher and uniform fluorescent signal. To achieve the maximum binding capacity, 50°C and the mole ratio of Tween-20 and succinic anhydride 1:1.5 and 10% carboxyl-Tween were chosen for the following experiments.

2.3 Identify the efficiency of capture probe immobilization

As shown in Figure 4, we compared the immobilization capacity of carboxyl derived-Tween coated epoxy beads, succinic anhydride modified amino beads and succinic acid modified amino beads, respectively. And the fluorescence signal from carboxyl derived-Tween coated epoxy beads is equal to succinic anhydride and DMAP modified amine-beads and succinic acid modified beads.

DMAP is a good example of a modern low-molecular organic catalyst with a powerful effect on many reactions including acylations on nitrogen as well as oxygen and carbon [24]. So we added a catalytic amount of DMAP in our acylation reaction. Amino-silanized beads were reacted with 10% succinic anhydride and DMAP (catalytic amount) in DMF solution under N₂ gas for 4 h with continuous shaking. And we found that with addition of DMAP, the higher immobilization capacity was achieved. Also we modified amino beads with succinic acid, with addition of catalyst EDC, and the high immobilization was achieved. And our carboxyl derived-Tween coated epoxy beads have the same high immobilization capacity, comparing to the optimized succinic anhydride modified amino beads and succinic acid modified amino beads. The immobilization capacity was also higher than normal 2D surface modification. Carboxyl derived-Tween coated epoxy beads would have potential value.

2.4 Specific binding using goat IgG, BSA, human IgG and rabbit IgG

We immobilized goat anti-rabbit IgG on Tween-coated beads. And a small sample of rabbit IgG, human IgG, goat IgG and BSA, respectively, was incubated with the beads. Due to the specific recognition between IgG and its corre

Figure 4 Detection of immobilized DL488 anti-rabbit IgG on succinic anhydride modified silica beads, succinic anhydride with catalyst DMAP modified silica beads, succinic acid modified silica beads, carboxyl derived-Tween modified silica beads, respectively.

sponding anti-rabbit IgG antibody, anti-rabbit conjugated beads would interact with corresponding rabbit IgG. Then a strong fluorescence signal could be observed after incubation with DL488 anti-rabbit IgG. As demonstrated in Figure 5, the results revealed that only rabbit IgG bond selectively to the beads and in the case of goat IgG, human IgG and BSA, the fluorescence signal was almost the same as background signal of the glass slide which the beads were dropped on the surface for signal detection. Aslo, due to Tween being a hydrophilic molecule and a small molecular weight, nonspecific protein adsorption is very low.

2.5 Sensitivity and dynamic range

Sandwich immunoassay was fabricated to detect rabbit IgG. And the concentration series varied from 100 ng/mL down to 50 pg/mL. As shown in Figure 6, as low as 50 pg/mL rabbit IgG could be detected, which is sufficiently low for the current clinical diagnostic applications. The result clearly showed that the fluorescence signal intensity was linear in a broad concentration range.

Hydroxyl groups and hydrophilic chain on the surface endow the beads with good water solubility. Thus, Tween modified beads with good hydrophilic performance reduce the likelihood for hydrophobic interactions as a potential cause for aggregation and protein non-specific adsorption and beads adsorption on centrifuge tube wall. So we could achieve high productivity and the low limit of detection. Besides, the homogeneous water environment could help to keep proteins in their active states.

3 Conclusion

We have developed a novel carboxyl derived-Tween coated suspension array technique for biomolecule detection. In this study, we have only described the design and fabrication of derivatized Tween-coated silica beads for protein

Figure 5 Specific recognition between antibody and antigen and non-specific protein adsorption on carboxyl derived-Tween coated epoxy particles.

Figure 6 Quantitative sandwich assay (a) diagram of the fluorescence intensity as a function of rabbit IgG concentration in the sandwich immune fluorescence assay. Fluorescence microscope images of Tween-coated beads: (b1) optical image; (b2) fluorescence image.

detection. And we have detected IgG using sandwich immune assay, the detection limit of rabbit IgG is as low as 50 pg/mL when using 5000 beads for analysis, which is sufficiently low for the current methods. In the future, we wish Tween modification could be used for detecting other biomolecules and clinical samples. Also our system could be used for multiplex assay, such as self-coded suspension based-silica beads. Therefore, further efforts will focus on the use of this modification for multiplexed assay. Our results demonstrated the potential use of Tween for modification in practical biotechnology and medical applications in various biodetection systems.

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