Preparation of Porous Protein-Based Hydogel for Highly Sensitive Protein Chips

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Summary: Protein chips are important tools for high-throughput analysis of biological events. We have developed a novel method to prepare a protein-based hydrogel, that is, a ''Three-Dimensional Nano-structured Protein Hydrogel'' (3-D NPH), which is composed of protein and polymer nano-particles. The 3-D NPH could be easily prepared by dispensing a protein and polymer mixture on a substrate. Surprisingly, gold particles conjugated with protein A diffused into the 3-D NPH which was made of mouse IgG through the pores. We have shown that the protein chips made with our 3-D NPH method has tremendously improved sensitivity in detecting protein-protein interactions compared with that of direct protein immobilization methods.

Keywords: high sensitivity; hydrogel; nano-particle; protein chip; protein immobilization

Introduction

Multiplex assays represented by the conventional immunoassay are essential for the analysis of biological events. Recently, in this field, protein chips have become important tools for the high-throughput analysis of biological events.[1–3] Protein chips have advantages: For example, we can analyze a number of protein-protein interactions at the same time. We also need only a small sample volume to measure these interactions. However, the signal of the interactions detected with these methods decreases, because detection areas for immobilization of bio-samples are smaller. Therefore, there has been a lot of researches to increase the signal of these interactions. One of such efforts is to immobilize a protein in a three dimensional manner. There has been a lot of researches on protein immobilization methods.[4] We summarize those methods in Table 1. The method of the immobilization to 2-D surface is the most popular, in which protein molecules are directly bound to a substrate in 2 dimensional condition, and hereafter we call this 2-D method. On the other hand, in other 3 methods, protein molecules are immobilized 3 dimensionally. Using these three methods, the amount of immobilized ligand proteins could be increased on the surfaces of the substrate comparing with 2-D method. However, the increase in the amount of immobilized ligand proteins has not necessarily achieved an increase in the amount of analyte proteins bound to ligand proteins.[5,6] These results could be explained by a reduced accessibility of analyte proteins to immobilized ligand protein, sometimes referred to as ''mass transfer limitation''. Therefore, conventional methods can not realize the increase in the signal of protein-protein interactions.

Our objective is to increase the sensitivity of protein chip. To do this, we have developed a novel method of protein immobilization. To increase the sensitivity of protein chips, we could make the composite gel composed by protein and reactive polymer mixture. Using our

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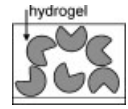
Table 1.
Comparison with conventional methods of protein immobilizations.

	Immobilization to 2 D surface	Immobilization of cross-linking	Immobilization into hydrogel	immobilization with polymer chains
				
Binding mode of immobilization	Physical adsorption Covalent binding	Covalent binding	Physical adsorption Covalent binding	Physical adsorption Covalent binding
State of immobilized proteins	2 D	3 D	3 D	3 D
Surface density of immobilized proteins	Low	High	High	High
Access of large analyte molecules	–	No	difficult	difficult

method, ligand proteins could be immobilized three dimensionally, and the composite gel had the unique porous structure enough to diffuse the analyte into it. The analyte reactivity of our 3-D NPH method increased compared with 2-D method. Therefore, we can achieve a significant improvement in the sensitivity of protein-protein interactions.

Materials and Methods

Our method can be overviewed in Figure 1. 3-D NPH was prepared by dispensing protein and reactive polymer mixtures on limited areas of substrates followed by a simple drying process. First, the protein and polymer solutions were mixed, then deposited on a substrate, which had succinimide groups, and concentrated through the drying process. After the activation with EDC/NHS on microarray-size slide, the mixture of protein and Poly(*N*-acryloylmorpholine-co-*N*-acryloxysuccinimide) (poly(NAM-co-NAS), protein: polymer = 10:1 (w/w) in buffer solution) was deposited on substrates which had the succinimide groups, and subsequently reacted through the drying process. Next, during the drying process, protein and polymer nano-

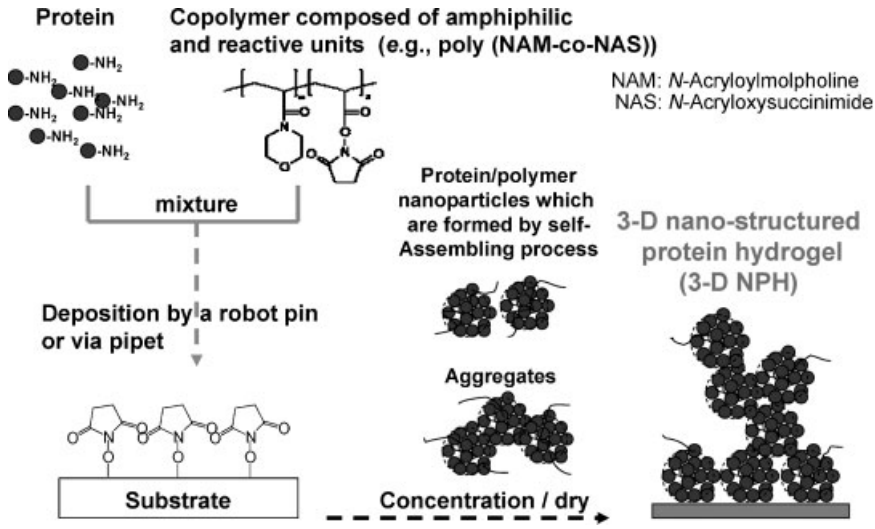


Figure 1.
Preparation method of 3-D NPH.

particles were formed by a self-assembling process. Finally, the nano-particles formed the three dimensional structure and construct a hydrogel on the substrate. These procedures to prepare 3-D NPH were carried out at room temperature. After about 1hr reaction, the remaining succinimide groups were masked by 1M ethanolamine solution (pH 8.5) and the substrates were washed with PBST buffer solutions 5 times. A typical polymer, used in our method, was a random co-polymer, which was composed of both amphiphilic and reactive units. We have confirmed that it is possible to use a large variety of polymers for this method.

Analysis of the Structure of 3-D NPH

In order to observe the cross section of 3-D NPH, we made a thick layer of gel. The 3-D NPH could be easily prepared by condensing mouse IgG antibody protein and poly (NAM-co-NAS) mixture on substrates. The SEM image shows the cross-section of 3-D NPH-mouse IgG (Figure 2). The thickness of 3-D NPH-mouse IgG was about 5 μm. As we can see in the SEM image, 3-D NPH-mouse IgG had a structure consisting of particles, approximately 100nm in size, that were made of proteins and polymers. In addition, we confirmed that the amino acid analysis revealed 3-D NPH was made of more than 90% of protein. The result showed that the large amount of proteins was immobilized on limited areas three-dimensionally. We discovered that 3-D NPH has a novel porous structure composed of protein-based nanoparticles.

In order to confirm whether the large molecules could diffuse into 3-D NPH, we observed TEM image of a 3-D NPH-mouse IgG cross section after staining 3-D NPH-mouse IgG with protein A - gold particles. The diameter of Protein A – gold particle is about 10nm. This 3-D NPH-mouse IgG was made of mouse IgG antibody protein. The TEM image revealed that protein A - gold particles certainly diffused into 3-D NPH-mouse IgG and bound specifically to the Fc regions of mouse IgG molecules immobilized in 3-D NPH-mouse IgG (Figure 3). Through the pores and spaces in 3-D NPH, gold particles carrying protein molecules could easily diffuse inside the

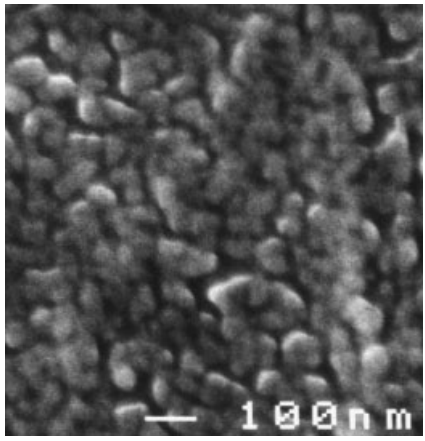


Figure 2.
SEM image of the cross section of 3-D NPH-mouse IgG. White scale bar is 100 nm.

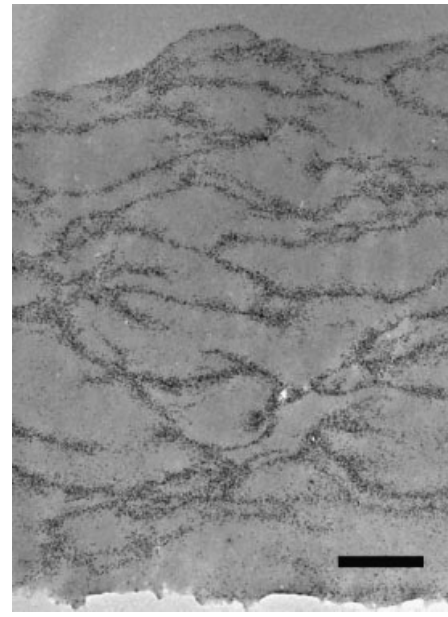


Figure 3.
TEM image of the cross section of 3-D NPH-mouse IgG after staining with protein A gold particles. The scale bar is 1 μm. Black lines in the cross section described protein A gold particles.

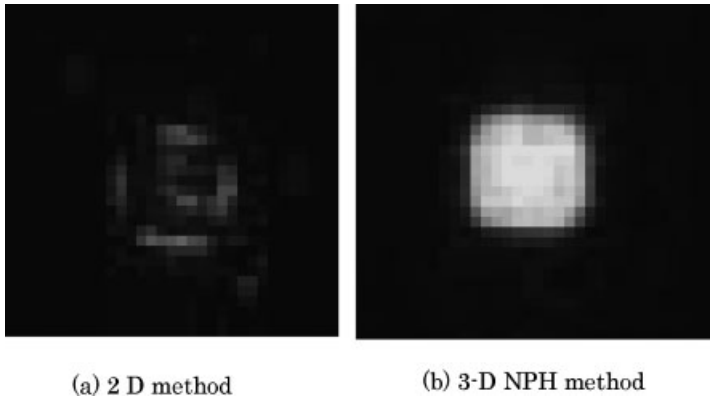


Figure 4.
Fluorescent image of 2 D method (a) and 3-D NPH method (b) after probing with Cy3 anti-mouse IgG F $(ab')_2$

3-D NPH. From this result, we concluded that 3-D NPH had sufficient pores to allow large molecules, such as analyte proteins, to diffuse into the hydrogel. Also, the immobilized ligand proteins retained their activities to interact with analyte proteins. Therefore, we expected that the protein chip made with 3-D NPH method showed tremendous improvement of protein-protein interactions compared with that made with the conventional method of protein immobilization onto the substrate.

Measurement of the Sensitivity of Protein-Protein Interaction with 3-D NPH

To confirm the sensitivity of the protein-protein interactions binding to the 3-D NPH, we made the 3-D NPH using mouse IgG antibody protein, and analyzed the interactions with Cy3 anti-mouse IgG $F(ab')_2$ as the analyte proteins using fluorescent measurement. On this case, we selected 2-D method as reference for the comparison. The concentration of Cy3 - anti-mouse IgG $F(ab')_2$ applied to the 3-D NPH-mouse IgG and 2-D surface was 10 μg/mL. As a result, the signal on the 3-D NPH-mouse-IgG was remarkably increased comparing with 2-D method (Figure 4). Therefore, we could increase the sensitivity using 3-D NPH comparing with the conventional methods. In addition, we confirmed that non-specific adsorption of anti-pSA IgG to 3-D NPH-mouse IgG was not detected. This result suggests 3-D NPH has good specificity of the protein-protein interactions. Therefore, our technology is suited for protein chip measurements.

Conclusion

We have developed the 3-D NPH method of protein immobilization for protein chip. This method enables ligand protein to form three dimensional self-assemblies on a substrate. The three dimensional structure has sufficient space to allow large molecules, such as analyte proteins, to diffuse into the hydrogel. We can achieve a significant improvement in detecting the sensitivity of protein-protein interactions using 3-D NPH method. In addition, the whole process of the proposed method is well suited to the mass production of protein chips.

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