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Gold-Nanoparticle-Modified Polyvinylidene Fluoride Membranes Used for Western Blotting with High Sensitivity

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In this study, we report an ultrasensitive western blotting method by introducing gold nanoparticles (AuNPs) onto modified polyvinylidene fluoride (PVDF) membranes via atom transfer radical polymerization (ATRP). AuNPs can prevent protein or peptide transfer across the PVDF membrane to reduce target loss. The results suggest that the improved western blotting method can be used to detect target biomarkers with satisfactory limit of detection (LOD). [1]

Western blotting, which is easy to use, inexpensive, semi-quantification, and specific identification of proteins, has become an indispensable tool and a widely used method for detecting target proteins in complex biological samples.[1] However, the effective detection of low-molecular-weight proteins and peptides remains a challenge because of the large pore diameters of the membranes relative to the size of the detected samples; furthermore, the weak binding strength of the samples results in the loss of material during the electro-transfer step and washing procedure.[2]

To overcome these challenges, several improvements have been made to western blotting. Nevertheless, these strategies do not entirely overcome the loss of small proteins and do not remarkably improve the detection sensitivity for proteins/peptides under 10 kDa.[3] The separated proteins need to be transferred to a membrane, such as a PVDF or nitrocellulose (NC) membrane, from the sodium salt polyacrylamide gel electrophoresis (SDS-PAGE) gel to facilitate the access of molecular probes. In addition, SDS-PAGE gels are difficult to dispose of in subsequent detection steps. PVDF or NC membranes are produced by microphase separation and are commercially available for western blotting. However, NC membranes are mechanically weak under repeated probing and exhibit poor high-temperature stability and poor resistance to acids, bases, and solvents. Therefore, PVDF membranes are primarily used because of their good retention capacity and excellent mechanical strength under acidic or basic conditions. [4]

In this research, AuNPs [5] were bonded to PVDF membranes via an ATRP reaction to develop a sensitive western blotting method for the detection of small-sized proteins. As an excellent nanomaterial, AuNPs exhibit numerous advantages, such as a high surface-to-volume ratio, facile combination with biological molecules without compromising biological activity, and availability in a wide range of sizes (1–200 nm).[6] The ATRP reaction, as an excellent conjugation method, can generate large branches at the PVDF membrane surface and greatly increase its surface area. Briefly, in the first step, a PVDF membrane was prepared and modified through an ATRP reaction. In the second step, AuNPs were prepared and introduced onto the branches of the PVDF membrane surface. When a test object was transferred to the membrane, the force between the test object and the AuNPs prevented it from passing through the PVDF membrane. Thus, the loss of small biomolecules was reduced and the sensitivity of detection (SOD) was improved.

In this study, AuNPs were prepared according to previous reports, with slight modifications.[7] Briefly, all glassware used in the experiments was soaked with aqua regia (one part HNO_3, three parts HCl) for 48 h, rinsed in doubly distilled water, and oven-dried prior to use. To prepare the AuNPs, $HAuCl_4 \cdot 4H_2O$ was added to doubly distilled water and brought to a boil under continuous stirring. Sodium citrate solution was then quickly added, and the resulting reaction mixture was stirred and kept boiling for 15 min. The solution color changed to a final color of wine-red during this period. After the boiling was sustained for 15 min, the heat source was removed; the suspension was then stirred for another 15 min and subsequently stored in dark bottles at 4°C until use.[8] The AuNPs were prone to aggregation and were therefore freshly prepared before use. A TEM image of the AuNPs is shown in Fig. 1.

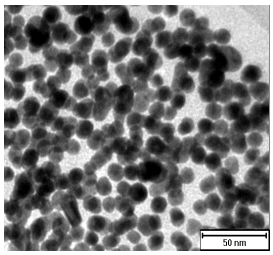

Fig. 1 TEM image of the prepared AuNPs.

Because of the low surface energy and strong hydrophobicity of PVDF membranes, their applications are greatly restricted. Therefore, hydrophilic modification of PVDF membranes is necessary before they are used, and the first step is preparation of the initiator. Briefly, 11-mercapto-

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1-undecanol was first dissolved in tetrahydrofuran (providing the reaction environment). Pyridine was subsequently added as a catalyst, and nitrogen gas was used in place of air. After the 11-mercapto-1-undecanol solution was cooled in an ice bath for 30 min, 2-bromoisobutyryl bromide was slowly added under a continuous flow of nitrogen. After the initiator mixture was stirred for 4 h, filtered, and dried under nitrogen, the initiator preparation was complete, and the initiator was stored at 4°C under nitrogen until use.

Surface-graft modification of PVDF membranes involves the use of physical or chemical reactions to generate active sites on the molecular chain of the membrane material under appropriate conditions. Target molecules are easily attached to the molecular chain of the material upon contact, which can effectively reduce further migration inside the membrane and significantly reduce the loss of small molecules. Research has shown that a PVDF membrane combined with AuNPs can effectively improve the load capacity of biological molecules.

PVDF-ATRP was prepared as follows. First, the initiator and the PVDF membrane were mixed together and placed on a rotating instrument overnight. After the membrane was washed 3 times with methanol, the ATRP reaction mixture and glucose were added to the membrane and shaken at room temperature (RT) for 24 h. After being gently washed 3 times, the PVDF-ATRP was stored at 4°C until use.

The modified PVDF membrane not only retained its excellent mechanical properties and stability under high temperatures, acidic and alkaline conditions, and other chemical environments but also exhibited improved hydrophilicity and overall performance.

The AuNPs were coated onto the PVDF membrane according to a traditional method. Briefly, the PVDF-ATRP and AuNPs were placed on a rotating instrument overnight. After the membrane was washed and centrifuged, the supernatant was discarded. Finally, after an additional centrifugation step at 13 300 rpm for 10 min, the preparation of the PVDF-ATRP-AuNPs was complete. Fig. 2 shows SEM images of commercial PVDF membrane and PVDF-ATRP-AuNPs membrane.

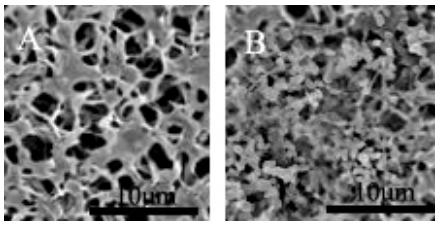

Fig. 2 SEM images of a commercially available PVDF membrane (A) and a modified PVDF-ATRP-AuNPs membrane (B).

As shown in Fig. 2, a large amount of gold nanoparticles were loaded onto the PVDF membrane surface after modification, and the number of active sites for binding target biomolecules was greatly increased.

The load of AuNPs on the membrane was measured using the following Eq. (a):

$$Load\ (\%) = (m-m_0)/m_0 \times 100\% \quad \text{(a)}$$

Where m_0 is the mass of the PVDF-ATRP membrane, and m is the mass of the PVDF-ATRP-AuNPs membrane modified with AuNPs.

In our experiments, we used an amino acid fragment (20-100) from hepatic erythropoietin (HPO) with a molecular weight of approximately 8.8 kDa as a model. First, the proteins were separated using SDS-PAGE as a matrix according to their molecular weights or three-dimensional structures. Second, the separated proteins were transferred to a PVDF membrane using electrophoretic transfer. Here, we used a PVDF membrane modified with AuNPs in place of the initial commercial PVDF membrane. The next steps involved blocking, antibody incubation, and detection of the protein. To ensure the transfer effects, the following steps should be noted. First, the transfer was performed by placing the transfer kit on ice to suppress heat generation. Second, the washing was gentle and slow to avoid disruption of the AuNPs attached to the PVDF membrane. The experimental procedures for this study are shown schematically in Fig. 3.

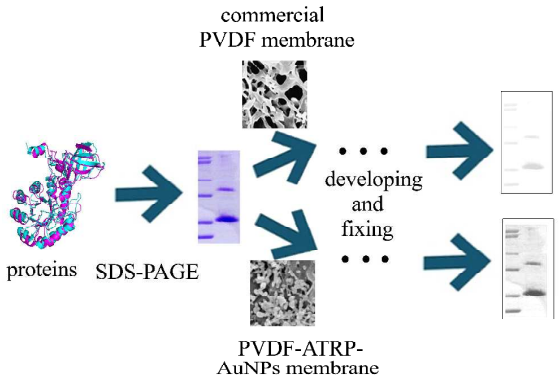

Fig. 3 Schematic diagram of western blotting using two different membranes.

To examine the performance of the newly modified PVDF membrane, the following experiment was conducted using the fragment of HPO as a model. First, the PVDF membrane was cut to a size of 6 cm × 8 cm for western blotting after being prepared according to the previously described steps. Western blotting was subsequently performed using this membrane. A commercial PVDF membrane was used for the control experiments, and the experimental results were compared. The signal on the membrane is directly related to the amount of target molecules detected, allowing the amount of target molecules on the PVDF membrane to be determined from the intensities of the grayscale spots after the membrane was developed and fixed.

The western blotting results indicate that the retention efficiency was increased nearly 20% in the PVDF-ATRP-AuNPs membrane compared with the conventional PVDF membrane. The improvement resulted from the introduction of AuNPs as a medium for target protein capture. Briefly, in conventional western blotting, small proteins or peptides are

apt to pass through the PVDF membrane with continued current after they have been transferred to the PVDF membrane because the pore sizes of the PVDF membrane are large compared to the small biomolecules. In the case of the improved PVDF-ATRP-AuNPs membrane, the AuNPs prevented the small biomolecules from passing through the membrane because of the attractive force between the AuNPs and the molecules. This increased biomolecule retention is advantageous because the retention of more biomolecules in the membrane results in improved detection signals.

In addition, modification of the PVDF membrane with the AuNPs did not influence its performance, and the AuNPs did not affect the subsequent developing and fixing steps.

After using the PVDF-ATRP-AuNPs membrane in a western blotting method with a fragment of HPO as a model, we demonstrated the application of the membrane for the detection of NSPc1. NSPc1 is a new gene of the polycomb group (PcG) family and was first isolated and identified in mouse embryonic development in 2001. NSPc1 expression is significantly elevated in many malignancies, and it can inhibit the expression of the p21 WAF1/CIP1 gene from the CDKI family as an apparent inhibitory factor. Thus, it plays an important role in the regulation of tumor cell proliferation and in differentiation processes. In this study, amino acids 110-190 were selected as the detection fragment, giving a molecular weight of approximately 8.8 kDa. The experimental procedures for this study are nearly the same as those used in conventional western blotting. In Fig. 4, a modified PVDF membrane and a commercial PVDF membrane are compared in terms of their signal intensity in response to gradually decreasing concentrations of sample from spot A to spot E.

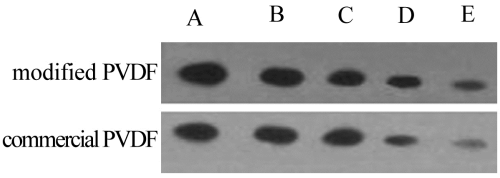

Fig. 4 Images of western blots using the PVDF-ATRP-AuNPs membrane and a commercial PVDF membrane.

Table 1 Grayscale values and the signal increase.

	A	B	C	D	E
PVDF-ATRP-AuNPs membrane	41.79	42.28	42.80	44.74	65.94
commercial PVDF membrane	71.87	59.53	51.80	78.04	111.20
signal increase (%)	41.85	28.98	17.37	42.67	40.70

As shown in Fig. 4, all the spots (from A to E) in the western blotting analysis using the modified PVDF membrane provides larger and more molecules intense compared to the corresponding commercial membrane. As evident from the results in Table 1, the grayscale was substantially improved in the modified PVDF membrane compared with that in the commercial PVDF membrane. This result indicates that the improved PVDF membrane increased the retention ratio of small molecules and that the detection sensitivity was distinctly improved. This result also suggests that use of the improved PVDF-ATRP-AuNPs membrane in real sample detection, such as in human serum, is feasible. In addition, the results show that the modified strategy owns good reproducibility.

We successfully modified a conventional PVDF membrane by introducing AuNPs *via* an ATRP reaction. In the grayscale comparison study, the PVDF-ATRP-AuNPs membrane demonstrated its greater ability to prevent small molecule penetration across the membrane. The acting force between the AuNPs and the biomolecules reduces the pass-through ratio greatly and increases the detection signal of the target protein or peptide. This modification did not require additional equipment, and the cost of the AuNPs was low. We believe that this improvement of PVDF membranes can enable new applications.

To our knowledge, this work represents the first time that ATRP modification has been used to study membrane permeability. The results shown here indicate the potential for using ATRP modification and nanomaterials to study membrane permeability and the interactions between bimolecules and membranes, which are important for further improving the performance of membranes and, more importantly, for developing membranes with different uses.

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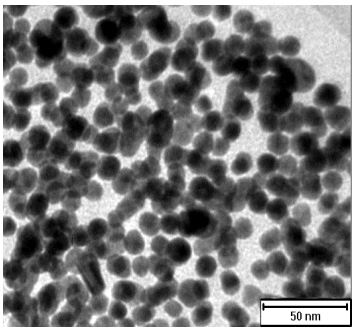


Fig. 1. TEM image of the prepared AuNPs.

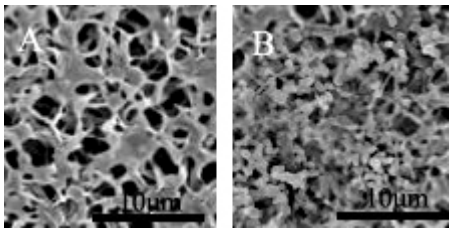
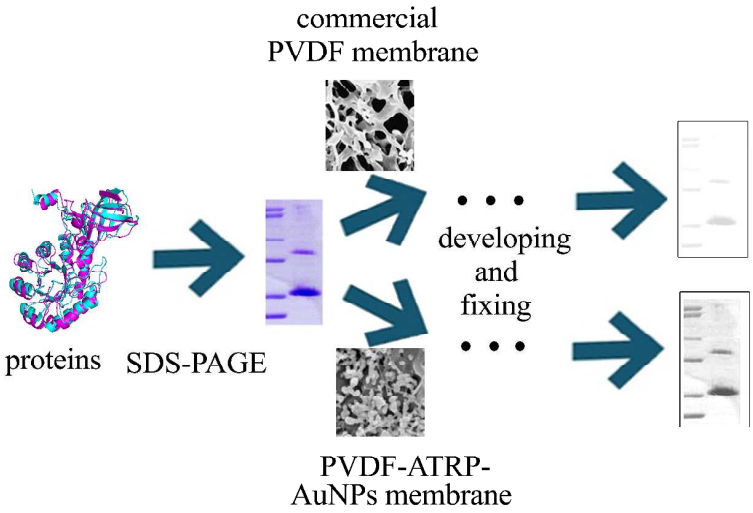


Fig. 2. SEM images of a commercially available PVDF membrane (A) and a modified PVDF-ATRP-AuNPs membrane (B).



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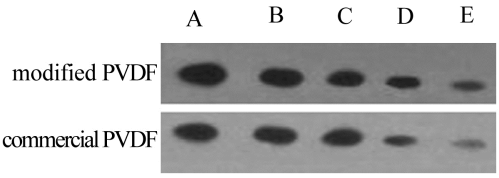

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