Urimem, a membrane that can store urinary proteins simply and economically, makes the large-scale storage of clinical samples possible

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Abstract

Biological samples from patients are invaluable for both medical research and medical practice. Ideally, the samples should be preserved for the same period of time as the duration of their corresponding medical records. Urine is a body fluid that can be non-invasively acquired, and it contains important biological information about the patient. Unlike blood which has mechanisms to keep the internal environment homeostatic, urine is more likely to reflect changes of the body. In other words, urine is likely to be a better biomarker source than blood. Here, we propose a method to adsorb urinary proteins onto a polyvinylidene fluoride (PVDF) membrane called Urimem. The method is very simple and inexpensive and requires minimal sample handling. It does not use organic solvents, and it is environmentally friendly. The proteins on the membrane are dried and stored in vacuum bag, which keeps the protein pattern faithfully preserved. The membrane may even permit storage at room temperature for weeks. The quantity of eluted proteins from the membrane is sufficient for biomarker validation experiments. Using this simple and inexpensive urinary protein preservation method, it is possible to begin preserving urine samples from all consenting patients. Thus, medical research especially biomarker research can be conducted more economically, ultimately benefiting the patients who provided the samples. This sample storage approach can facilitate the biomarker research and potentially change the landscape of medical research and medical practice.

Introduction

Biological samples from patients are invaluable for both medical research and medical practice. However, biological samples from patients are not currently preserved as comprehensively or as long as their corresponding medical records, primarily because of the invasiveness, difficulty and cost associated with collecting and storing such samples. Urine is a body fluid that can be non-invasively acquired, and it contains important biological information about the patient from whom it was obtained. Urinary proteins are considered to be the best resource of potential biomarkers for kidney disorders. There is no other example of a non-invasively accessible body fluid that is so closely associated with a vital organ. Furthermore, because urine is the filtrate of the blood produced by the kidney, urinary proteins can provide not only detailed information about the urinary system but also information about the blood, which reflects the condition of the whole body. Unlike blood which has mechanisms to keep the internal environment homeostatic, urine is more likely to reflect changes of the body. In other words, urine is likely to be a better biomarker source than

blood [1].

Therefore, urine is an important biological sample that should be preserved for each stage of a disease for all patients. The preservation of a large number of urinary samples for validation is a critical step that facilitates biomarker research and the translation from the laboratory to the clinic. The preservation of urine is commonly performed by freezing the urine and storing it at -80 ℃. Due to its large volume and low protein concentration, the storage of urine often requires a significant amount of space. Furthermore, the freezing of urine cannot absolutely prevent the degradation of the urinary proteome. Urinary proteins are more easily degraded in liquid than under completely dry conditions.

Simple and inexpensive urinary protein sample preservation can be the starting point for long and comprehensive biological sample storage. Here, we propose a method to directly adsorb urinary proteins from the urine onto a polyvinylidene fluoride (PVDF) membrane that can then be dried and stored. This method is very simple and inexpensive and requires minimal sample handling. It does not use organic solvents and is environmentally friendly. More importantly, the proteins that are bound to the membrane are dry, which prevents their degradation and makes their preservation at room temperature for longer times possible. Because PVDF membranes have a limited protein-loading capacity, the most important consideration is that the proteins in a given urine sample are all adsorbed into the PVDF membrane to faithfully preserve the protein pattern.

Materials and Methods

Ethics Approval

This study aims to establish a method that uses PVDF membranes to preserve urinary proteins. In this methodology study, only normal human urine was collected naturally from volunteer students in our lab. As human waste, no invasive measures were taken. All participants provided verbal informed consent to allow us to use their urine samples for this study. No written consent was provided because none of participants thought it was necessary. The verbal consent was documented in the laboratory notebook of the authors. The verbal consent procedure and research protocol in this study were approved by the Medical Ethics Committee of Peking Union Medical College (Project No:018-2013).

Urinary protein preservation on the membrane

1. Determine the urinary protein concentration from a previous routine urine test. In case of proteinuria, the urinary protein concentration needs to be determined and the urine needs to be diluted to the normal human urinary protein concentration of less than 100mg/L.

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Prepare 47 mm diameter medium-speed qualitative filter paper and PVDF membranes (Immobilon-PSQ Membrane, PVDF, 0.2 μm, 26.5 cm x 3.75 m roll; one PVDF membrane mapping to 4-6 sheets of filter papers).

2. Set the thermostatic centrifuge to 12,000×g and the temperature to 4 ℃. Centrifuge the diluted urine samples for 10 min and save the supernatant.

Optional: Pass 20 ml diluted urine sample through a 0.45 μm filter membrane (Millipore Durapore membrane filters, filter type: 0.45 μm HV) with ultra-low protein-binding capacity and save the flow-through.

3. Place 4-6 sheets of wetted circular filter paper onto the vacuum suction filter bottle (10 cm^2 filter area).

4. Immediately place one activated PVDF membrane onto the filter paper (before using the PVDF membrane, ensure that it has been activated in methanol and rinsed with pure water) while being careful to avoid the generation of bubbles.

5. Install the vacuum suction filter bottle and fill it with 20 ml supernatant or the flow-through from the 0.45 μm filter membrane.

6. Connect the vacuum suction filter bottle to the vacuum pump, and allow the solution to pass through the PVDF membrane drop-wise by adjusting the vacuum pressure to approximately 7 kPa. The initial velocity should be approximately 1.3 droplets/second, and the flow rate should decrease until the solution stops dripping. Turn off the vacuum pump. The total filtration time should be approximately 4 min.

7. After the proteins are adsorbed onto the PVDF membrane, the protein-bound membrane is placed under a bulb with 1100 W (275 W*4) of power for 3 to 4 min to allow drying to completion.

Optional: The protein-bound membrane is allowed to dry to completion at room temperature.

8. Place the dry membrane with tag paper into aseptic sealing membranes. Keep the tag paper and dry membrane separate by sealing the membrane between them. Sealing the membrane with Vacuum packaging machine to keep the dry membrane stored in vacuum. Then they were stored at -80 ℃. When other people save urine proteins using this method, the tag paper should contain a unique number, which is used to find the information of this sample, and all the information of this sample (recorded information: medical record number, date and time urine was collected, before or after taking drugs, routine urine test number, etc) should be stored in the computer.

Urinary protein elution from the membrane

The elution buffer was composed of 1% Triton X-100 and 2% SDS in 50 mM Tris-HCl,

pH 9.5 [2]. Briefly, the protein-bound dry membrane was cut into small pieces and placed in a clean tube, to which 0.1 ml elution buffer/cm^2 membrane was added. The membrane in the elution buffer was mixed well by first vortexing for 10 min at room temperature and then by ultrasound for 15 min in an ultrasonic cleaner at room temperature. The supernatant was collected by spinning down the membrane. The protein can be precipitated with chloroform/methanol if the detergent needed to be removed for downstream analyses, such as protein quantification and LC-MS/MS analysis.

Results

Testing the loading capacity of the PVDF membrane

As shown in Figure 1, 30 µl elution buffer was separated by SDS-PAGE (12% gels) and stained with coomassie brilliant blue after eluting the proteins from the PVDF membrane with 1 ml elution buffer. The centrifuged urine with different volumes was passed through 10 cm^2 PVDF membranes and the filtrates were passed through new sheets of PVDF membrane. After the protein-bound membrane was dried to completion in room temperature, the proteins were eluted from the membrane with 1 ml elution buffer. 30 µl elution buffers were used for SDS-PAGE analysis. Lanes 1, 3, 5, 7, and 9 represent the eluted proteins from 10 ml, 20 ml, 30 ml, 40 ml, and 50 ml of urine, respectively, from 5 different sheets of 10 cm^2 PVDF membrane. Lanes 2, 4, 6, 8, and 10 represent the eluted proteins from the filtrates of 10 ml, 20 ml, 30 ml, 40 ml, and 50 ml of urine. For urine volumes greater than 30 ml, the proteins were not entirely adsorbed onto the membrane. Thus, the proportion of urine volume and the PVDF membrane area was determined to be 20 ml/10 cm^2, which permits the urine proteins almost completely preserved on the PVDF membrane at this condition. Aliquot of this urine sample was concentrated by centrifugal filter with a molecular weight cut-off of 3,000 Da. The protein concentration of concentrated urine was measured by the Bradford protein assay. After calculation, the protein concentration of this urine sample was 33 ug/ml. Therefore, the amount of urine proteins that was preserved on the PVDF membrane was 66 µg/cm^2 in this experiment.

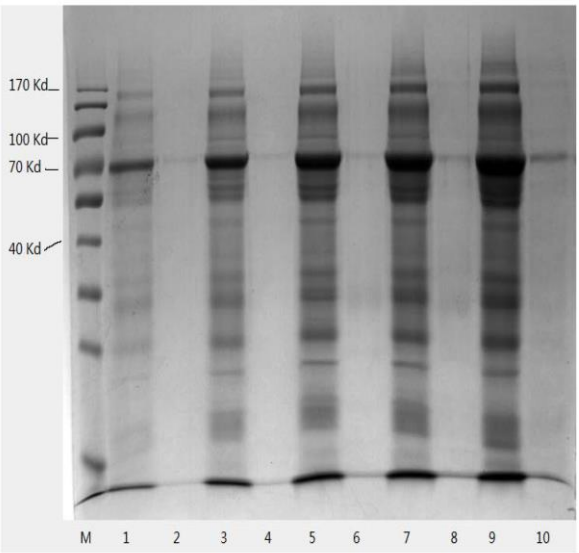

Figure 1. Testing the loading capacity of the PVDF membrane using SDS-PAGE analysis.

After protein elution from the PVDF membrane with 1 ml elution buffer, 30 µl elution buffer was used for SDS-PAGE. Lanes 1, 3, 5, 7, and 9 represent the eluted proteins from 10 ml, 20 ml, 30 ml, 40 ml, and 50 ml of urine, respectively, from 5 different sheets of 10 cm2 PVDF membrane; The filtrates of 10 ml, 20 ml, 30 ml, 40 ml, and 50 ml of urine were passed through new sheets of 10 cm2 PVDF membrane, respectively. Lanes 2, 4, 6, 8, and 10 represent the eluted proteins from the filtrates of 10 ml, 20 ml, 30 ml, 40 ml, and 50 ml of urine.

The urinary proteins recovered from the membrane preserved for 18 days at -80 ℃ and at room temperature exhibit the same SDS-PAGE pattern

Four 20 ml aliquots of urine were passed through 4 sheets of 10 cm^2 PVDF membranes and stored at four temperature conditions for 18 days, including room temperature, 4 ℃, -20 ℃, and -80 ℃. After protein elution from the PVDF membrane with 1 ml elution buffer, 30 µl elution buffer was separated by SDS-PAGE (12% gels) and stained with coomassie brilliant blue. The proteins that were stored at -80 ℃, -20 ℃, 4 ℃, and room temperature are shown in lanes 1, 2, 3, and 4 of Figure 2, respectively. The urinary proteins stored at different temperature exhibit similar SDS-PAGE pattern.

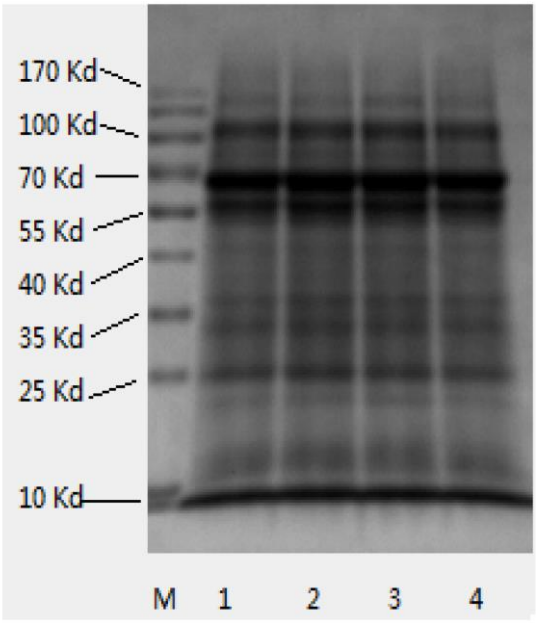

Figure 2. Comparing the urinary proteins recovered from the membranes after preservation for 18 days at different temperatures.

Four 20 ml aliquots of urine were passed through 4 sheets of 10 cm2 PVDF membranes and stored for 18 days at four temperature conditions: room temperature, 4 ℃, -20 ℃, and -80 ℃ for 18 days. After protein elution from the PVDF membrane with 1 ml elution buffer, 30 µl elution buffer was analyzed by SDS-PAGE. The proteins that were stored at -80 ℃, -20 ℃, 4 ℃, and room temperature are shown in lanes 1, 2, 3, and 4, respectively.

Discussion

As difficult as it is to believe today, a single concept developed by Dr. Henry Plummer at the beginning of the 20th century changed the face of medicine. This concept involved a centralized medical record that was stored in a single repository and capable of traveling with the patient [3]. This concept is also applicable to the field of biological sample preservation. Comprehensive biological sample storage may change the face of medicine again. Urinary proteins provide rich biological information of the body especially those changes we called biomarkers. Without the homeostasis mechanisms, urine is more likely to be the gold mine of biomarker research. This investigation is the first study to report the use of a PVDF membrane to preserve urinary proteins. Urine proteins from as much as 20 ml urine can be preserved onto 10 cm^2 PVDF membrane in five minutes or less. Stored dry and in vacuum, the membrane prevents protein degradation and facilitates sample transfer. It should be noted that nitrocellulose membranes (NC membranes) can also be used to preserve urine proteins following this method.

Proteins that are preserved on PVDF/NC membranes are compatible with traditional downstream analytical applications. First, proteins on the membrane can be stained using all commonly used protein stains, such as Ponceau-S Red, Coomassie Brilliant Blue R dye, and Amido Black, enabling the quantification of the total amount of protein on the membrane [4-6]. A second potential application is the immunodetection of the proteins on the membrane by dot blotting. Third, the preserved proteins can be eluted from the membrane for other applications, such as western blotting or LC-MS/MS analysis [2,7].

This simple and inexpensive urinary protein preservation method makes it possible to begin preserving urine samples from all consenting patients during each stage of disease development. However, several considerations must be taken into account when preserving urinary protein samples. A sample taken at a certain time point should be well documented in the patient's medical record. Patient consensus may be required at the time the sample is taken and when the sample is analyzed as part of a particular study. As the concept of urinary protein storage is gradually accepted by the medical community, technical standards will likely be developed, and commercial products will likely be produced. It is likely that many new technologies will be developed, including more durable media with improved protein adsorption capabilities; test strips to estimate protein quantity; streamlined protocols for urinary protein collection, drying, sealing, packaging and labeling; sample storage and management systems for individual sample access and retrieval; and an optimal manner in which to use the membrane-adsorbed proteins. Storage at 4 ℃ or even ambient temperatures for longer time periods may become feasible. The use of particular resins might allow small molecules, including creatinine and certain ions, to be stored economically in the future. Other body fluids, such as cerebrospinal fluid, can also be stored using the same approach.

Comprehensive historical biological information can also be used in retrospective studies to understand the pathophysiologies of certain diseases and potential relationships among diseases or to monitor the long-term efficacies and side effects of treatments. There will be more ways to extract and utilize the information, providing that an increased number of samples become available for research. With this information, medical research can be conducted easier, faster, and more economically, ultimately benefiting the patients who provided the samples.

We believe that it is now possible to begin preserving urinary protein samples from each stage of disease development for every consenting patient in hospitals. This could potentially change the current landscape of medical research and medical practice.

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