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

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By nature, biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood which has mechanisms to minimize changes and to keep the internal environment homeostatic, urine is more likely to reflect changes of the body and is a better biomarker source. Because of its potential in biomarker discovery, urinary proteins should be preserved comprehensively as the duration of the patients' corresponding medical records. Here, we propose a method to adsorb urinary proteins onto a membrane we named Urimem. This simple and inexpensive method requires minimal sample handling, uses no organic solvents, and is environmentally friendly. Urine samples were filtered through the membrane, and urinary proteins were adsorbed onto the membrane. The proteins on the membrane were dried and stored in a vacuum bag, which keeps the protein pattern faithfully preserved. The membrane may even permit storage at room temperature for weeks. Using this simple and inexpensive method, it is possible to begin preserving urine samples from all consenting people. Thus, medical research especially biomarker research can be conducted more economically. Even more objective large-scale prospective studies will be possible. This method has the potential to change the landscape of medical research and medical practice.

urine proteins, biological-sample preservation, PVDF/NC membrane

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Biological samples from patients are invaluable for both medical research and clinical practice. However, such samples are not currently preserved as comprehensively or for as long as their corresponding medical records, primarily because of the invasiveness, difficulty and cost associated with their collection and storage. By nature, biomarker is the measurable change associated with a physiological or pathophysiological process (http://blog.sciencenet.cn/blog-244733-725226.html). 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, as an important biomarker source, should be preserved for each stage of the disease for all patients. The preservation of a large number of urinary samples for validation is a critical step that facilitates biomarker research and its translation from the laboratory to the clinic. The preservation of urine was commonly performed by freezing the urine and storing it at –80°C; however, this requires a significant amount of space because of its large volume and low protein concentration. Furthermore, the

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freezing of urine cannot absolutely prevent the degradation of the urinary proteins since 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 for directly adsorbing urinary proteins onto a polyvinylidene difluoride (PVDF) membrane that can then be dried and stored. This method is simple and inexpensive and requires minimal sample handling, avoids the use of organic solvents and is environmentally friendly. Importantly, the proteins bound to the membrane are dry, which prevents their degradation and allows them to be preserved at room temperature for longer periods. Because PVDF membranes have a limited protein-loading capacity, it is important to ensure that the proteins in a given urine sample are all adsorbed onto the membrane, in order to preserve the protein pattern faithfully.

1 Materials and methods

1.1 Ethical approval

This study aimed to establish a method using PVDF membranes to preserve urinary proteins. Urine was collected from volunteer students in our laboratory. No invasive measures were required, and all participants provided verbal informed consent to allow their urine samples to be used for this study. Written consent was not considered to be necessary, but the record of verbal consent was documented by the authors in the laboratory notebook. The verbal consent procedure and research protocol were approved by the Medical Ethics Committee of Peking Union Medical College (Project No. 018-2013).

1.2 Urinary protein preservation on the membrane

A flow chart explaining the method is shown in Figure 1. The procedure was carried out as follows. (i) The urinary protein concentration was determined from previous routine urine tests. In the case of proteinuria, the urinary protein concentration was determined and the urine was diluted to a normal human urinary protein concentration of <100 mg L^{-1}. The filter paper used was 47-mm diameter medium-speed qualitative filter paper, and the PVDF membranes were Immobilon-PSQ Membrane (PVDF, 0.2 μm, Millipore, Headquartered in Billerica, Massachusetts). (ii) The diluted samples were centrifuged in a thermostatic centrifuge for 10 min at 12000×*g* and 4°C and the supernatant was saved. As an optional stage, 20 mL diluted urine sample was passed through a 0.45-μm filter membrane (Durapore membrane filters, filter type: 0.45 μm HV, Millipore, Headquartered in Billerica, Massachusetts) with ultra-low protein-binding capacity and the flow-through was saved. (iii) Four to six sheets of wetted circular filter paper were placed onto the vacuum suction filter bottle (10-cm^2 filter area). (iv) One activated PVDF membrane (activated in methanol and rinsed with pure water) was placed immediately onto the filter paper, avoiding the generation of bubbles. (v) The vacuum suction filter bottle was installed and loaded with 20 mL supernatant or the flow-through from the 0.45-μm filter membrane. (vi) The vacuum suction filter bottle was connected to the vacuum pump, and the solution allowed passing through the PVDF membrane drop-wise, by adjusting the vacuum pressure to approximately 7 kPa, with an initial velocity of approximately 1.3 droplets s^{-1}. The total filtration time was approximately 4 min. (vii) After the proteins were adsorbed onto the PVDF membrane, the protein-bound membrane was placed under four 275 W bulbs for 3–4 min to complete the drying process, or left to dry at room temperature. (viii) The dry membrane with tag paper was placed between aseptic sealing membranes to keep the tag paper and dry membrane separate. The membrane was then sealed using a kitchen vacuum sealer and stored at −80°C. The tag paper contained a unique number, which could be cross-referenced with other information relevant to the sample (medical record number, date and time urine was collected, before or after taking drugs, routine urine test number), and stored on the computer.

1.3 Urinary protein elution from the membrane

The elution buffer was composed of 1% Triton X-100 and 2% sodium dodecyl sulfate (SDS) in 50 mmol L^{-1} 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

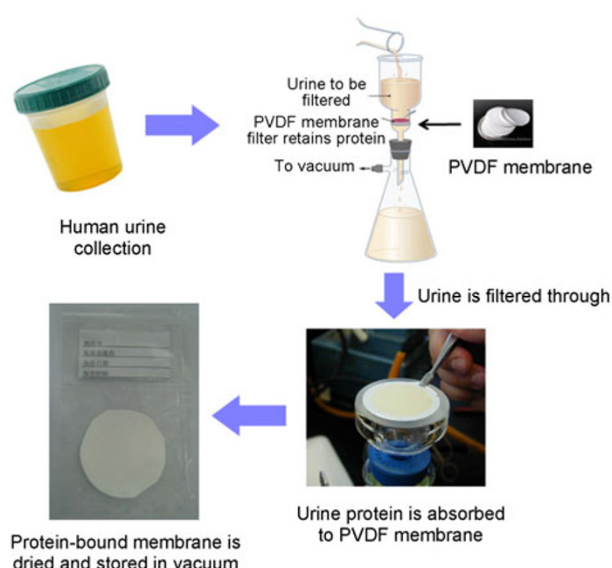

Figure 1 The flow chart of this method.

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 could be precipitated with chloroform/methanol if the detergent needed to be removed for downstream analyses, such as protein quantification and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

2 Results

2.1 Loading capacity of the PVDF membrane

Different volumes of centrifuged urine were passed through 10-cm^2 PVDF membranes and the filtrates were then passed through new PVDF membranes to detect proteins not adsorbed by the first membrane. The protein-bound membranes were dried completely at room temperature, and the proteins were eluted using 1 mL elution buffer. Equal volumes (30 μL) of elution buffer were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gels) and stained with Coomassie Brilliant Blue (Figure 2). Comparison of the first and second adsorptions of 20 mL urine each (20 F and 20 S, respectively) revealed that most of the proteins were adsorbed on the membrane at the first adsorption. Thus 20 mL urine per 10 cm^2 membrane allowed almost complete preservation of urinary proteins on the PVDF membrane under these conditions. The protein concentrations of the urine samples were about 33 μg mL^{-1}, equivalent to 66 μg cm^{-2} in this experiment.

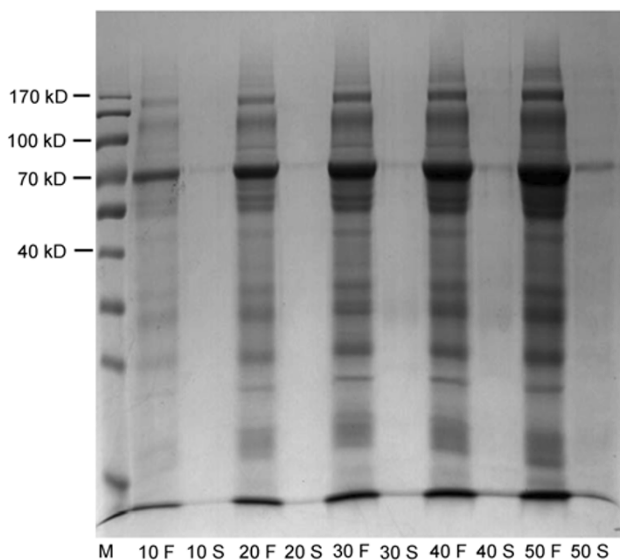

Figure 2 The loading capacity of the PVDF membrane was tested using SDS-PAGE analysis. The numbers refer to the volume of urine used in mL. F refers to protein eluted from the first membrane adsorption; S refers to protein eluted from the second membrane adsorption (e.g., 20 S=proteins eluted from second membrane adsorption of 20 mL urine). Most of the proteins were adsorbed on the membrane during the first adsorption.

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

Four 20-mL aliquots of urine were passed through four 10-cm^2 PVDF membrane sheets and then stored under four different temperature conditions for 18 d: room temperature, 4°C, −20°C, and −80°C. Proteins were eluted from the PVDF membranes with 1 mL elution buffer, separated in 30 μL elution buffer by SDS-PAGE (12% gels) and stained with Coomassie Brilliant Blue. Urinary proteins stored at different temperatures exhibited similar SDS- PAGE patterns (Figure 3).

3 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, and comprehensive storage of biological samples could further revolutionize medical research and practice. Urinary proteins provide rich biological in-

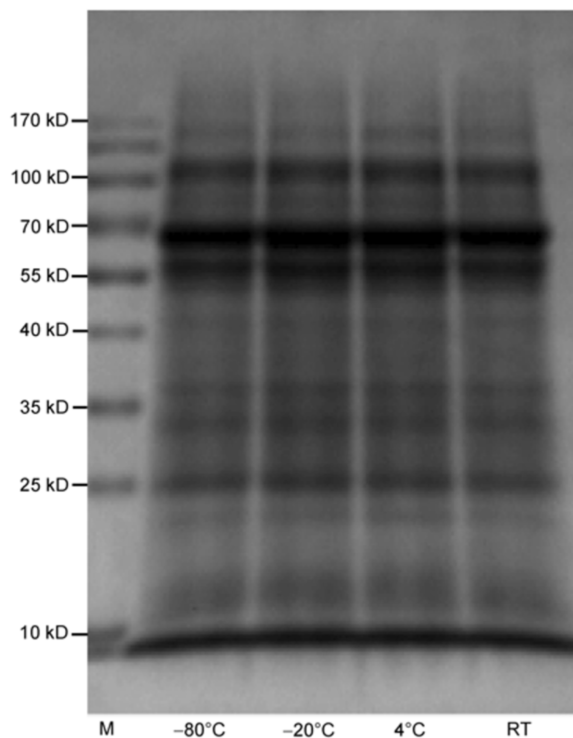

Figure 3 Urinary proteins recovered from membranes after preservation for 18 d at different temperatures. Four 20-mL aliquots of urine were passed through four 10-cm^2 PVDF membrane sheets and stored for 18 d at four temperatures: room temperature (RT), 4°C, −20 °C, and −80°C. After protein elution from the PVDF membrane with 1 mL elution buffer, 30 μL elution buffers were analyzed by SDS-PAGE.

formation of the body especially those changes we called biomarkers. Without the homeostasis mechanisms, urine is more likely to preserve the changes and become the gold mine of biomarker research. The current study was the first to report the use of a PVDF membrane to preserve urinary proteins. Urinary proteins from as much as 20 mL urine were preserved on 10-cm^2 PVDF membrane within 5 min or less. The membranes could be stored dry in a vacuum bag, thus preventing protein degradation and facilitating sample transfer between institutions. Samples could also be preserved using nitrocellulose (NC) membranes, using a similar method.

Proteins preserved on PVDF/NC membranes can subsequently be analyzed using traditional downstream analytical applications. Adsorbed proteins can be stained with all commonly-used protein stains, such as Ponceau-S Red, Coomassie Brilliant Blue R dye, and Amido Black, enabling quantification of the protein on the membrane [4–6]. Proteins on the membrane can also be subjected to immunodetection by dot blotting, and 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 method of preserving urinary proteins makes it possible to begin preserving urine samples from all consenting patients during each stage of disease development. However, several factors need to be considered. Samples taken at certain time points should be well-documented in the patient's medical record, and patient consent may be required, both when the sample is collected, and when it is analyzed as part of a particular study. Once urinary protein storage becomes an accepted practice by the medical community, technical standards and commercial products are likely to be developed. New technologies may emerge, 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 optimal procedures for the use of membrane-adsorbed proteins. Storage at 4°C or even at ambient temperatures for longer periods may become feasible, while the use of resins might make the preservation of small molecules, including creatinine and certain ions, an economic prospect. Other body fluids, such as cerebrospinal fluid, can also be stored using the same approach.

Comprehensive historical biological information can also be used in both prospective and retrospective studies to improve our understanding of the pathophysiology of certain diseases and potential relationships among diseases, or for monitoring the long-term efficacies and side effects of treatments. More ways of extracting and using the information will become evident as increasing numbers of samples become available for research. This information will make medical research easier, faster, and more economic, ultimately benefiting the patients who provided the samples.

We believe that the current technique provides a practical method for preserving urinary protein samples from consenting people during every stage of the disease development. Even more objective large scale prospective studies will be possible. This procedure has the potential to change the current landscape of medical research and medical practice.

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