Chapter 3
Urimem Facilitates Kidney Disease Biomarker Research

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Abstract Urine is a body fluid that can be noninvasively acquired and contains important biological information about the patient. Urinary proteins are considered to be the best resource of potential biomarkers for kidney disorders. Urinary proteins can be adsorbed to polyvinylidene fluoride (PVDF) or nitrocellulose membranes, which can then be dried and stored in vacuum bag. This membrane is named Urimem. The membrane can even be stored at room temperature for at least weeks without changing the quantity of eluted proteins. With this simple and inexpensive urimem, it is possible to begin preserving urine sample from all consenting patients during each stage of kidney disease development. Thus, the medical research can be conducted more economically, ultimately benefiting the patients who provided the samples. This can potentially change the landscape of medical research and medical practice.

Keywords Urimem · Storage · Urinary proteins

Hard as it is to believe today, a single concept developed by Dr. Henry Plummer at the beginning of the twentieth century changed the face of medicine. The concept

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was a centralized medical record, stored in a single repository, and capable of traveling with the patient [1].

In this century, comprehensive biological sample storage may change the face of medicine again. 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, expenses, and usefulness that are associated with collecting and storing such samples. Urine is a body fluid that can be noninvasively acquired and 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 noninvasively 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.

Therefore, urine is an important biological sample that should be preserved for each stage of a disease for each patient. The preservation of a large number of urinary samples for validation is a key step that facilitates biomarker research and the translation from the laboratory to the clinic.

The preservation of urine is commonly performed by freezing and storing it at −80 °C. Due to its large volume and low protein concentration, the storage of urine usually requires a significant amount of space. Urinary proteins may be degraded easier in solution than in complete dry condition.

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 absorb urinary proteins on 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 environment-friendly. More importantly, the proteins that are bound on the membrane are dry, which prevents their degradation and makes their preservation at room temperature for longer time possible. Because PVDF membranes have a limited protein loading capacity, the most important consideration is that the proteins in one urine sample are all adsorbed into the PVDF membrane to keep the protein pattern faithfully preserved [1].

3.1 Urinary Protein Preservation on the Membrane

1. Determine the urinary protein concentration from a previous urine routine test. Save a portion of the urine for a new urine routine test.
   - — No dilution and 3× dilution;
   - ± 3× and 6× dilution;

+	6× and 30× dilution;
++	30× and 60× dilution;
+++	60× and 120× dilution;
++++	120× and 240× dilution;

Prepare 47-mm-diameter medium-speed qualitative filter paper and PVDF membranes (Immobilon-PSQ Membrane, PVDF, 0.2 μm, 26.5 cm × 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 °C. Centrifuge the diluted urine samples for 10 min and keep 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 retain the flow-through.
3. Place 4–6 sheets of wetted circular filter paper on the Vacuum suction filter bottle (10 cm^2 filter area).
4. Place one activated PVDF membrane on the filter paper immediately (before using the PVDF membrane, ensure that it has been activated in methanol and cleaned in 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 dropwise 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 being adsorbed to the PVDF membrane, the protein-bound membrane is placed under a bulb with 1,100 W (275 W × 4) of power for 3–4 min to allow drying to completion.
   Optional: The protein-bound membrane is placed in room temperature condition and let it dry to completion naturally.
8. Place the dry membrane with tag paper (recorded information: medical record number, date, and time urine was collected, before or after taking drugs, urine routine test number) into two independent aseptic sealing membranes and store at −80 °C.

Urinary protein elution from the membrane The elution buffer comprised 1 % Triton X-100, 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 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 was concentrated using a centrifugal filter with a molecular weight cutoff of 3,000 Da or by protein precipitation with

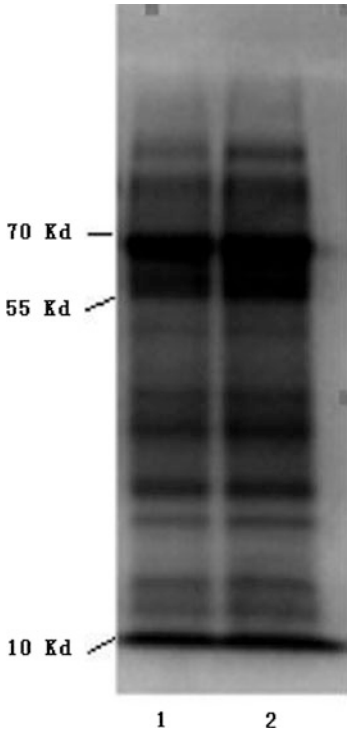

Fig. 3.1 Comparing the urinary proteins after removing cells in urine by centrifugation or filtration, respectively [1]

chloroform/methanol (if the detergent needed to be removed for the downstream analysis such as protein quantification and LC-MS/MS analysis).

Centrifugation or filtration to remove cells in urine As shown in Fig. 3.1, after protein elution from the PVDF membrane with 1 ml elution buffer, 30 μl elution buffer was used for SDS-PAGE. Urine samples that were either passed through a 0.45 μm filter membrane with ultra-low protein binding capacity or centrifuged to remove cells are shown in lanes 1 and 2, respectively. Using a 0.45-μm filter membrane with an ultra-low protein binding capacity conveniently retains cells from the urine sample on the membrane.

Testing the loading capacity of the PVDF membrane As shown in Fig. 3.2, 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, 20, 30, 40, and 50 ml of urine, respectively, in 5 sheets of 10 cm^2 PVDF membrane. Lanes 2, 4, 6, 8, and 10 represent the eluted proteins in the flow-through from the PVDF membrane onto which 10, 20, 30, 40, or 50 ml of urine proteins was absorbed from another 5 sheets of 10 cm^2 PVDF membrane. For urine volumes greater than 30 ml, the protein was not absorbed entirely into the membrane. Thus, the largest urine volume was 20 ml/10 cm^2, and the urine protein concentration was

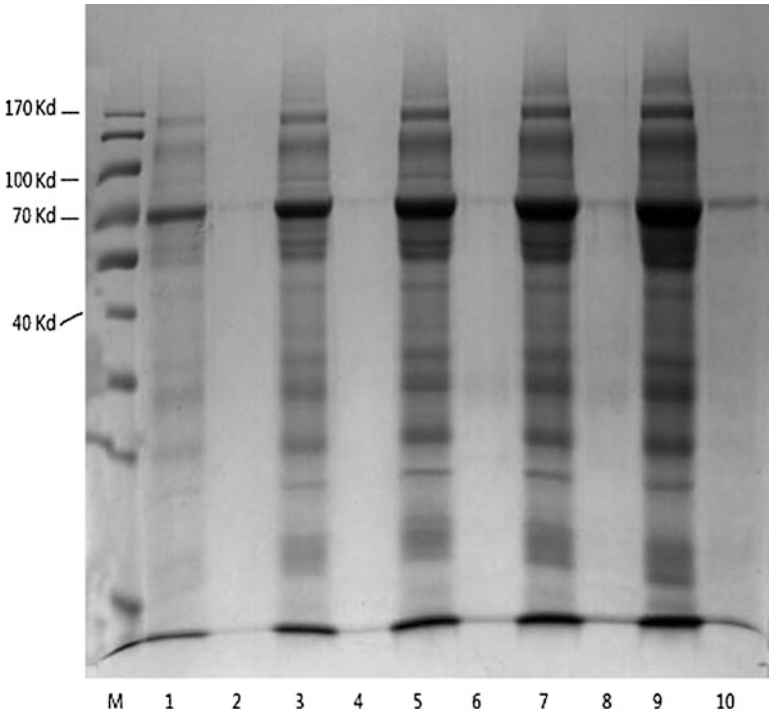

Fig. 3.2 Testing the loading capacity of the PVDF membrane using SDS-PAGE analysis [1]

33 μg/ml. The concentration of the urine protein that passed through the PVDF membrane was less than 66 μg/cm^2.

The urinary proteins recovered from the membrane preserved for 18 days at −80 °C and at room temperature exhibit the same SDS-PAGE pattern Four aliquots of 20 ml of urine proteins were retained on 4 sheets of 10 cm^2 PVDF membranes and stored at four temperature conditions: room temperature, 4, −20, and −80 °C for 18 days. After protein elution from the PVDF membrane with 1 ml elution buffer, 30 μl elution buffer was used for SDS-PAGE. Shown in lanes 1, 2, 3, and 4 of Fig. 3.3 are the proteins that were stored at −80, −20, 4 °C, and room temperature, respectively.

Twenty milliliters of nondiluted proteinuria and 20 ml of 20-fold-diluted proteinuria were filtered through 10 cm^2 PVDF membranes (pore size at 0.22 μm). Then, we precipitated the proteins by chloroform/methanol to remove the detergent. After re-solubilization with lysis buffer (7 M urea, 2 M thiourea, 120 mM DTT, 40 mM Tris-base) and quantification of the protein concentration, equal amounts of protein (25 μg) were separated via SDS-PAGE. As shown in Fig. 3.4, lanes 1 and 2 represent proteinuria and a 20-fold dilution of proteinuria, respectively. The protein patterns were consistent, and no differential bands were observed.

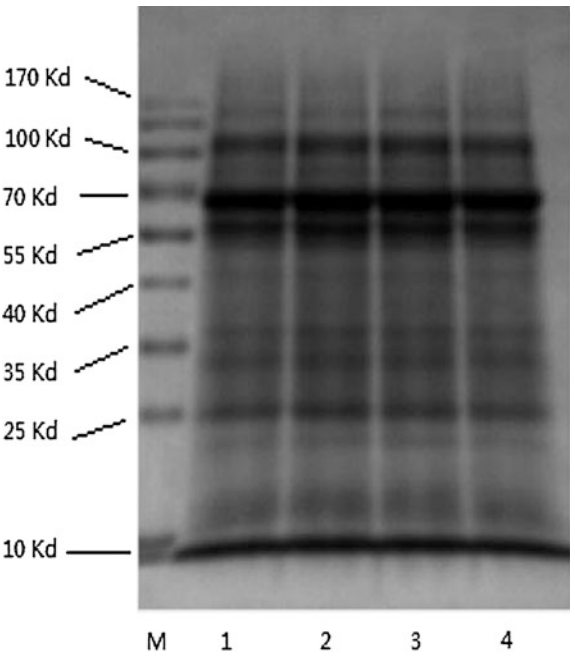

Fig. 3.3 Comparing the urinary proteins recovered from the membranes after preservation for 18 days with different temperature [1]

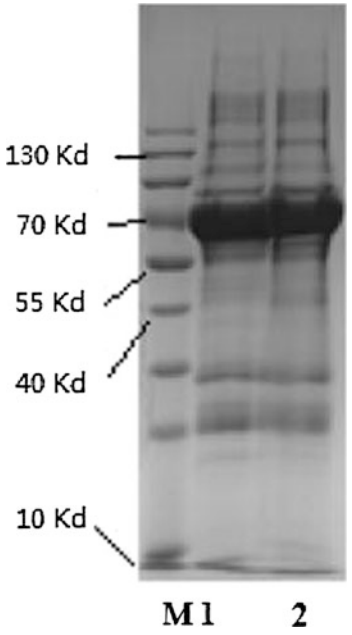

Fig. 3.4 Comparing the urine proteins recovered from the membranes that were loaded onto different amount of urine proteins [1]

This is the first study to report the use of a PVDF membrane to preserve urinary proteins to facilitate biomarker research. Proteins that are preserved on the PVDF membrane are compatible with traditional downstream analytical applications. First, proteins on the membrane can be stained by all commonly used protein stains, such as Ponceau-S Red, Coomassie Brilliant Blue R dye, and Amido Black. The proteins on the membrane can also be visualized by transillumination based on the change of the color of the PVDF membrane from opaque to semi-transparent when wet in 20 % methanol. Based on this result, the total amount of protein on the membrane can be quantified when used in conjunction with densitometry [3]. 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 and LC-MS/MS analysis.

By using this simple and inexpensive urinary protein preservation method, it is possible to begin preserving urine sample 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 that the sample is taken and also when the sample is analyzed as part of a particular study. As the concept of urinary protein storage is accepted gradually 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 protein. Storage at 4 °C or even ambient temperature for longer time periods may be feasible. The use of particular resins might allow small molecules, including creatinine and ions, to be stored economically in the future. Other body fluid such as cerebrospinal fluid can also be stored the same way. In addition, if the cellular structure of blood cells can be compromised, then blood can be blotted without any processing; thus, genetic information will also be available.

Comprehensive historical biological information can also be used in retrospective studies to understand the pathophysiology of disease and the relationships among diseases as well as to monitor the long-term efficacy and side effects of treatments. There will be more ways of extracting and using the information as long as more and more samples are available for research. With this information, medical research can be conducted more easily, considerably faster, and more economically, ultimately benefiting the patients who provided the samples.

We believe, starting from now, it is possible to start preserving urinary protein samples from each stage of disease development for every consenting patient in hospital. This can potentially change the landscape of medical research and medical practice of this century.

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