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Current state of the art for enhancing urine biomarker discovery

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Abstract

Urine is a highly desirable biospecimen for biomarker analysis because it can be collected recurrently by non-invasive techniques, in relatively large volumes. Urine contains cellular elements, biochemicals, and proteins derived from glomerular filtration of plasma, renal tubule excretion, and urogenital tract secretions that reflect, at a given time point, an individual's metabolic and pathophysiologic state. High-resolution mass spectrometry, coupled with state of the art fractionation systems are revealing the plethora of diagnostic/prognostic proteomic information existing within urinary exosomes, glycoproteins, and proteins. Affinity capture pre-processing techniques such as combinatorial peptide ligand libraries and biomarker harvesting hydrogel nanoparticles are enabling measurement/identification of previously undetectable urinary proteins. Future challenges in the urinary proteomics field include a) defining either single or multiple, universally applicable data normalization methods for comparing results within and between individual patients/data sets, and b) defining expected urinary protein levels in healthy individuals.

Keywords

biomarker; combinatorial peptide ligand libraries; diabetes; glycoprotein; hydrogel nanoparticles; Lyme Disease; mass spectrometry; protein; proteomics; selected reaction monitoring; urine

1. Introduction

"*The composition of the blood is determined not by what the mouth ingests but what the kidney keeps*." [1] This simple quote underscores the complex process of urine formation via plasma filtration by renal glomeruli, selective reabsorption/secretion of water, glucose, and ions from the renal tubules, and shedding of proteins from the kidney and urinary tract [1-3]. 30% of the urinary proteome is derived from glomerular plasma filtration and 70% of the urinary proteome originates from the urogenital tract; therefore the urine proteome is

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expected to contain a variety of potential biomarkers related to overall health as well as specific urogenital organ pathophysiology [1, 2].

Urine biochemicals such as glucose, ketones, and total protein have been routinely measureable for many years, however the unique protein/peptide composition of urine has been under-explored. Even though urine is generally available in milliliter quantities and can be collected frequently and non-invasively, urine proteomic analysis has lagged behind that of serum/plasma, despite blood's more complex protein repertoire [4]. For example, manual annotation of the literature by the UniProt consortium has revealed between 13,841 and 17,132 human protein-coding genes [5]. Yet, by 2015, only 4,430 urinary proteins had been identified, a large number of which were detected due to recent improvements in pre-analytical fractionation/concentration techniques [6, 7].

Physiologically, the urine proteome is less complex compared to the plasma proteome but this fact alone does not fully explain the smaller number of identified proteins. The dearth of information about the urine proteome/peptidome has been due to historically labor-intensive methods such as 2-D gel electrophoresis that fail to distinguish protein isoforms [8], poor precision of enzyme linked immunosorbent assays (ELISA) with urine [9], the variable urine matrix/proteome between and within individuals [10-14], the presence of high abundance proteins (e.g. uromodulin, albumin, alpha-1-antitrypsin, transferrin, immunoglobulin, alpha-1-microglobulin and complement) that mask low abundance proteins [15, 16], endogenous proteases [17], and a lack of technologies for rapid profiling of the urine proteome, with or without quantitative analyses (Table 1) [16].

However, in the past decade, analytical advances in quantitative mass spectrometry and fractionation techniques [18, 19], introduction of capillary electrophoresis mass spectrometry (CE-MS) [20, 21], and novel pre-analytical sample processing methods are advancing the field of urinary proteomics [22-25]. Several database repositories and urine proteomic resources are now available for locating information regarding urinary protein names, pathophysiology, literature citations, and consensus documents for analytical methods (Table 2). Many of the issues encountered in proteomic profiling and biomarker discovery, including sample preparation and pre-analytical variables [2, 26-31], interfering substances such as bacteria, antibiotics, and x-ray contrast media [32-34], the need for standardized procedures [35-39] and inter-laboratory comparisons [40], have been debated and extensively reviewed elsewhere [36, 41-45]. This review presents a snapshot of the current state of urinary protein biomarker discovery, with emphasis on pre-analytical processing and fractionation methodologies for improving the detection of low abundance proteins and/or protein classes, such as glycoproteins.

2. Summary of urinary proteomic biomarker discovery to date

Technological advances in proteomics and genomics are rapidly advancing our understanding of the molecular basis of disease. Translational research embodies these new diagnosis and treatment paradigms and is globally referred to as precision medicine, molecular medicine, or personalized medicine. The common goal, regardless of the name, is to design therapies based on a patient's phenotype and their genomic and/or proteomic

disease profile, coupled to our knowledge of biomarkers and prognostic factors [46, 47]. Urinary proteomics is a perfect example of the embodiment of precision medicine in which research is enhancing our assessment of disease risk, elucidating disease mechanisms, and predicting optimal therapy [48]. Extensive reviews of the translational research progress in the urinary proteomic field have been published [2, 41, 49-51]. Potential urinary biomarkers are being discovered in a variety of non-kidney associated diseases including acute appendicitis [52, 53], infectious diseases such as Tuberculosis [54], Chagas Disease and Lyme Disease [55-57], cancer [49, 50, 58-61], cardiovascular disease [62-64], and aging [65, 66]. Selected examples of the progress in urinary protein identification are described below, highlighting a) improvements in mass spectrometry-based urinary proteomic discovery, b) the number of identifiable proteins, and c) pathophysiologic associations over the last decade.

In 2001, Sphar *et. al.* established the efficacy of urine protein identification [16]. 124 proteins/expressed sequence tags were identified from unfractionated, pooled normal male urine by applying liquid chromatography coupled mass spectrometry (LC-MS) with iterative peptide ion analysis on a hybrid quadrupole-time-of-flight (Q-TOF) instrument. The combined abundance of 115 proteins represented less than 10% of the sample by mass, thus highlighting the magnitude of low abundance proteins in urine [16]. By 2006, linear ion trap-Fourier transform (LTQ-FT) and linear ion trap-orbitrap (LTQ-Orbitrap) mass spectrometers were being utilized for urine protein discovery due to the instruments' high resolution, mass accuracy, wide dynamic range, and fast cycle times. Adachi *et. al.* identified 1543 urinary proteins in 10 specimens from healthy individuals (one individual and a pool of 9 specimens) [67]. This study identified plasma membrane and lysosomal proteins in the urine, some of which are known to be associated with urinary exosomes, thus underscoring the complex source of urine proteins/peptides [68, 69].

Using high resolution Fourier transform mass spectrometry, Marimuthu *et. al.* discovered 1823 urine proteins, 671 of which were previously unreported in the urine, from a urine pool specimen comprising 24 healthy individuals [70]. A unique aspect of this study was lectin affinity chromatography with concanavalin A, wheat germ agglutinin and jacalin, to enrich glycoprotein constituents.

A comprehensive kidney, urine, and plasma proteome comparison was conducted by Farrah *et. al.* in 2013 [42]. By combining kidney, urine, and plasma datasets collected from different laboratories, with an estimate of relative protein abundance based on spectral counting and a normalization strategy to compare the proteomes, they were able to identify 2491 non-redundant proteins in the PeptideAtlas (www.peptideatlas.org/hupo/hkup) (Table 2) [71].

3. Advancements in mass spectrometry based urinary proteomics

The ever-increasing depth and breadth of the urinary proteome is attributable to the versatility of mass spectrometry for protein discovery, identification, relative and absolute quantification. Top-down protein profiling begins with a statistically significant number of complex biological samples which are separated by chromatography or 2-D gel electrophoresis. Specific fractions or gel spots containing proteins are analyzed in a mass

spectrometer (e.g. MALDI-TOF, SELDI TOF, or electrospray) to identify potential biomarkers. Top-down proteomics provides protein identity, relative quantity, and mass information for naturally occurring small peptides, post-translational modifications, and protein cleavages.

Bottom-up proteomic studies start with a few enzymatically digested protein samples to create a complex peptide mixture. The peptide mixture can be analyzed by liquid chromatography tandem mass spectrometry (e.g. LC-MS/MS or LC-MALDI MS/MS), providing high-resolution identity and relative quantity of many peptide sequences. Bottom-up approaches are low-throughput methods to analyze a few specimens (10 or less) from simple model systems such as treated/untreated cultured cell lines [18, 19, 72]. Specific details of mass spectrometry for proteomics are thoroughly described in the updated reviews by Bantscheff *et.al.* [18, 19]

Several different groups have developed tactics for enhancing identification and quantification of urinary proteins. A simple approach for improving urine protein detection was developed by combining silver staining with classic cellulose acetate membrane electrophoresis, prior to mass spectrometric analysis. Cellulose acetate membrane electrophoresis (CAME) is the classical method for identifying free light chain immunoglobulins (Bence-Jones proteins) in urine, based on relative mobility of the immunoglobulin fractions [73]. CAME allows discrimination of well-separated proteins on the cellulose acetate membrane, as well as the overlapping proteins. Nakayama *et. al.* have improved on the technique by incorporating a silver staining step immediately following electrophoresis to better fractionate the CAME protein patterns [74]. Well-separated protein bands are observed with <800mg/l total protein loaded per lane. Proteins are extracted over 8 hours in Laemmli sample buffer. The protein bands are excised from the cellulose acetate membrane and extracted for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and identified via mass spectrometry. This method successfully demonstrated the ability to identify 31 proteins from the urine samples of two tubulointerstitial nephritis patients. Of the 31 proteins identified, only 11 had been previously identified by immunofixation [74].

Protein post-translational modifications such as phosphorylation can indicate the activation state of cell signaling pathways [75]. Phosphoprotein enrichment is often performed using titanium dioxide chromatography during specimen processing [76]. Li *et. al.* developed two methods, Integrated Multidimensional Liquid Chromatography (IMDL) and Yin-yang multidimensional liquid chromatography (MDLC) tandem mass spectrometry, to identify urine proteins/phosphoproteins without using a titanium dioxide pre-fractionation step [77]. Although their method did not identify a significant number of previously unidentified urinary proteins, they were able to identify 1310 non-redundant proteins including 59 phosphorylation sites [77].

Following identification of potential proteomic biomarkers using top-down/bottom-up mass spectrometry, validation and absolute quantification of urine biomarkers is best achieved by Selected Reaction Monitoring (SRM) [35, 78, 79]. SRM, also known as Multiple Reaction Monitoring (MRM), allows previously defined peptides and fragment ions, known as

signature peptides, to be quantified by monitoring multiple fragment ions produced during collision-induced dissociation [18]. For robust SRM analysis with strong signal intensity, the peptides for SRM analysis must be unique to the protein of interest, should not contain post-translational modifications, and should not have genetically encoded variations [78]. Absolute quantification can be performed with stable isotope-labeled peptides corresponding to the signature peptides. The stable isotope-labeled peptide differs from the signature peptide only by mass, thus acting as an internal standard for comparison to the peptides of interest [79]. PeptideAtlas and PRIDE databases provide publically available lists of peptides suitable for SRM [18, 42, 71] (Table 2). Liquid chromatography SRM has been shown to be a robust method across laboratories, with a coefficient of variation (CV) <30% between 4 laboratories, when combined with pre-analytical enrichment strategies in serum, urine, and seminal fluid specimens [80].

SRM can be performed for specific peptide isoforms and post-translational modifications; however, the methods may require optimization due to potentially weak SRM signal intensity [18, 78]. Using uromodulin as a model protein, Fu *et. al.* provided details for an experimental workflow to identify trypsin-digested signature peptide fragments for use in SRM quantification assays [78]. Uromodulin (Tamm-Horsfall glycoprotein, 68 kDa), the most abundant urine protein, is produced by the ascending loop of Henle in the kidney [1, 81]. Uromodulin function is not completely understood, but it has been associated with renal calculi formation [82], hypertension [83, 84], and kidney disease [85, 86]. Furthermore, uromodulin peptide abundance has been shown by CE-MS to be increased in patients with pre-eclampsia [87] and rheumatoid arthritis [88], as well as with age [66]. However, uromodulin forms large aggregates, impeding typical mass spectrometry analysis. Using pooled healthy urine specimens and specimens from participants in the Atherosclerosis Risk in Communities study of 1987-1989, Fu *et. al.* identified 4 signature uromodulin peptides for SRM analysis that exhibited linearity and correlation to uromodulin protein abundance over 3 orders of magnitude [78]. Their process required extensive optimization due to complexities linked to composition of the urine sample matrix, biological characteristics of the target protein, and chromatography and spectroscopy incompatibility with data-dependent MS testing. Their workflow included: a) Identifying potential peptides within the detectable mass/charge (m/z) range, b) Refining candidate peptides based on a peptide length of 6-21 amino acids, c) Eliminating readily oxidizable peptides and peptides prone to post-translational modifications, d) Eliminating peptides only found in specific isoforms, unless the specific isoform was the intended target for quantification, e) Creating a correlation matrix to identify peptides that did not appear in chromatograms in a predictable fashion, and f) Selecting signature peptides that correlated well with each other, under a multitude of experimental conditions [78]. Signature peptides of uromodulin were identified that illustrated strong correlation of abundance ($r^2 > 0.90$) with each other and with the target protein within a complex milieu of pooled urine samples. The results were comparable to those derived by ELISA and demonstrated significant statistical superiority to data-dependent MS screening methods and unfocused SRM quantification techniques [78].

Cantley *et. al.* developed a workflow, termed the Targeted Urine Proteome Assay (TUPA), for evaluating a large number of urinary protein biomarkers [89]. To develop the assay, Cantley *et. al.* performed literature searches plus multiple urine proteomic assays from four

patients with immediate or delayed graft function following kidney transplantation. 416 potential biomarkers of kidney disease were identified [89]. The multiple proteomic assays included difference gel electrophoresis, iTRAQ, and label free quantitation in undepleted, high abundance protein depleted, and moderate abundance protein depleted samples. High abundance plasma proteins were depleted using a chicken polyclonal antibody column (Seppro® IgY14 LC10), with subsequent removal of moderately abundant plasma proteins on a Seppro® Supermix LC2 column [89]. The IgY antibodies in the Supermix LC2 column are directed against plasma proteins present in the flow-through of the LC10 columns [89]. Trypsin-digested peptides accounting for 200 of the proteins were identified, and synthetic, isotope labeled standards were assayed to eliminate peptides that exhibited deviation due to urine interference in MRM assays. The resulting TUPA assay featured 224 peptides corresponding to 167 proteins. However, a caveat of the TUPA assay is that the avian antibody depletion strategy removes plasma proteins, but not necessarily the highly abundant urinary protein, uromodulin, which may mask low abundance proteins. This plasma protein depletion strategy may explain their findings that 97 proteins from the high+moderate abundance depleted fractions did not show significant fold differences as they did in the non-depleted specimen [89].

Further methods are being developed to provide reproducible, high throughput, quantitative urinary proteomic data. In the classical data-dependent acquisition (DDA) method, LC-MS peak intensities of peptide precursor ions are subjected to fragmentation (sequencing) as they are eluted from the liquid chromatography system, without defining the peptides beforehand [18]. DDA is a sequential operation, analyzing one peptide at a time. However, under-sampling is a problem due to the molecular complexity and dynamic expression range of urine proteins [90]. An alternative method, similar to SRM, is data-independent acquisition (DIA) in which many peptides at a time are subjected to simultaneous fragmentation [18]. Peptide identification in DIA is performed by comparing the DIA chromatographic data with a peptide spectral library from previously identified proteins [91, 92].

To identify and quantify the majority of accessible urinary proteins without fractionating the urine specimens, Muntel *et. al.* have developed a data-independent acquisition (DIA) workflow in which a urinary spectral library is first created from a data-dependent acquisition (DDA) analysis of numerous urine samples using multiple MS/MS instruments [90]. In Muntel's study, urine from children diagnosed with ovarian cyst (n=12), urinary tract infection (n=11), and an abdominal pain control group (n=64) were analyzed by LC-MS/MS to create a spectral library [90]. A subset of these specimens (n=23) were analyzed in the same manner to create a second spectral library [90]. When combined with a publicly available human spectral library, a comprehensive spectral library of over 10,000 proteins was generated. Using a unique urine sample (unassociated with library development), a comparison of DIA and DDA analyses was performed. The results demonstrated a marked advantage for DIA, exhibiting a nearly two-fold increase in identified peptides and proteins, and a significant reduction in the coefficient of variation (CV) for quantification [90].

4. Urinary glycoproteomics

Urine contains glycosylated proteins, one of which is the highly abundant protein, uromodulin. Glycosylation is an abundant post-translational modification occurring on asparagine (*N*-linked) and serine/threonine (*O*-linked) residues [93]. Glycosylation determines protein sub-cellular location and trafficking to microdomains [94], mediates cell adhesion, functions as a receptor in cell-to-cell communication and immune response [95]. Lectins, which are carbohydrate binding proteins, can be utilized in affinity chromatography to procure glycoprotein enriched specimen fractions. Marimuthu *et. al.* utilized concanavalin A, wheat germ agglutinin, and jacalin to enrich for mannose, N-acteylglucosamine, and galactose/N-acetylgalactosamine containing glycoproteins, respectively [70]. Lectin-bound proteins were eluted using equimolar mixtures of N-acetylglucosamine, melibiose and galactose. The eluate was dialyzed against wash buffer to remove free sugars and concentrated using 3kDa cutoff filters prior to mass spectrometry [70]. Following separation of the proteins by SDS-PAGE, the gel bands were excised and digested with trypsin prior to protein sequencing by LC-MS/MS. Proteins in the lectin-enriched fraction were mapped to the Human Protein Reference Database (Table 2) to assess post-translational modifications, domains, and motifs [70]. This solid phase affinity capture method categorized proteins derived from sub-cellular locations, including cytoplasmic and endoplasmic reticulum associated proteins, as well as a group of voltage gated potassium ion channels [70]. Voltage gated potassium ion channels have been linked to cell migration, angiogenesis, adhesion and apoptosis, underscoring the important diagnostic information hidden in the urinary proteome [96].

The first targeted urinary glycoproteomics study characterized desialylated glycopeptides by tandem MS experiments utilizing collision induced dissociation and electron capture dissociation fragmentation techniques [93]. 58 *N*- and 63 *O*-linked glycopeptides were categorized from 53 glycoproteins [93].

Peptides and n-glycans in body fluids can be collected separately using a single GlycoFilter without additional purification [97]. This spin filter device allows separation and capture of glycans based on pH adjustment and filtration in a step-wise pre-processing, de-N-glycosylation, and proteolysis process [97]. By incorporating stable isotope labeled oxygen (^{18}O) into the peptide during enzymatic deglycosylation, the glycosylated peptide can be identified by the *N*-glycosylation consensus motif (Asn-XXX-Ser/Thr, in which XXX is any amino acid except proline) and the presence of ^{18}O asparagine [97].

Another important source of glycosylated proteins can be found in urinary exosomes. Saraswat *et. al.* examined urinary exosome glycosylation patterns because the exosome glycosylation pattern influences exosome targeting and uptake [98]. 37 glycoproteins were identified in urinary exosomes by comparing the urinary exosome glycan structures to a database rather than through iterative *de novo* glycan structure analysis [98]. Glycoproteins were digested with trypsin prior to enrichment by *Sambucus nigra* agglutinin (SNA) affinity chromatography or size exclusion chromatography. SNA binds preferentially to sialic acid attached to terminal galactose in α-2,6 and to a lesser degree, α-2,3 linkages. The glycoprotein enriched fractions were analyzed using collision induced dissociation-Tandem

MS [98]. From these glycoproteins, 126 *N*-glycopeptides from 51 *N*-glycosylation sites were characterized [98]. These studies highlight several different methods for enhancing identification of urinary glycoproteins.

5. Sources of variability in urine proteomics discovery

Despite technological improvements, pre-analytical variables continue to plague proteomic research. Pre-analytical variability creates barriers to inter-laboratory comparisons beyond the existing complicating factors of analytical platform differences and data reduction methods. Hydration status, diet, medications, age, and time of urine collection (first morning, second morning, random) are a few of the factors associated with inter and intra-patient urine biochemical and proteomic variability (Table 1) [11-14]. Urine specimen collection can be highly variable between studies and patient populations. Depending on the specimen requirements, collection site, and patient population (pediatric, neonatal, elderly, volunteers, healthy, etc), urine specimens may be collected via supra-pubic aspiration directly from the bladder, via an indwelling catheter, a specimen collection bag (for pediatric patients), or during voiding (mid-stream collection). The specimen collection container can also introduce variations depending on the container's construction, for example polypropylene, polystyrene, or glass, with/without a sterile interior. A “clean catch” specimen prevents excessive bacterial and cellular contaminates, however patient self-collected clean catch specimens are subject to variability in the collection technique [1]. To limit collection variability, the second morning urine specimen is typically collected, thus avoiding endogenous proteolytic activity during over-night urine retention in the bladder [17].

Urinary proteomic analysis is further complicated by differences in processing steps, such as ‘spin first’ methods to remove cellular elements by sedimentation, or ‘freeze first’ methods to stabilize proteins. Urine may contain a variety of cellular elements, organic and inorganic crystals and casts, collectively referred to as “sediment” or “pellet”. Red blood cells, leukocytes, renal and uroepithelial cells may be shed into the urine in various pathologic conditions. Urinary casts, a cylindrical structure formed in the kidney by precipitation of Tamm-Horsfall mucoprotein (uromodulin), with or without cells, lipids, or crystals, also contribute to urine sediment [99]. Freezing a urine specimen prior to centrifugation may cause cell lysis upon thawing, allowing cellular cytoplasmic proteins to contaminate the thawed urine specimen. On the other hand, spinning a specimen first removes the majority of intact cellular elements, thus depleting the proteome of these cell-derived proteins [28, 35]. Depending on the goal of the study and physiologic location of the potential biomarker (cellular versus soluble urine protein), the cellular content may be desirable or undesirable in the urinary biomarker analysis

Additional sources of pre-analytical variability introduced during urine specimen processing include centrifugation protocols (speed, time, temperature, brake settings), pH, salinity, protein concentration, and addition of protease inhibitors or chemical stabilizers [27-29, 35, 100, 101]. Urine fractionation strategies to deplete high abundance proteins and/or enrich low abundance proteins include solid phase adsorption, combinatorial peptide ligand libraries (CPLL), and hydrogel nanoparticle (NP) harvesting (Table 3) [89, 102, 103]. The

effectiveness of these fractionation strategies can be manipulated by varying the physicochemical properties of the system. Changes in salinity, protein concentration, ionic strength, and/or pH can alter protein-protein interactions by a) modifying adsorption to solid phase columns, b) altering the strength of hexapeptide-protein interactions, or c) by enhancing or inhibiting hydrophobic interactions between affinity baits and proteins, or between hexapeptides and complementary proteins [6, 25, 104-107]. For example, increased salt concentration can denature antibodies thus inhibiting solid phase affinity capture [105]. Decreasing the ionic strength, increasing hydrophobic interactions, and maintaining solutions within pH 4-9 of CPLL solutions can improve protein enrichment/depletion strategies using combinatorial peptide ligand libraries (discussed further below) [6, 107].

In summary, specimen collection, processing and fractionation schemas should be determined for each study prior to urine collection and processing. Clinical data and specimen processing details should be associated with each specimen, particularly for specimens obtained from a biorepository, because these clinical factors can help identify potential sources of variability and provide potential parameters for normalizing urinary biomarker data [108].

6. Determining the normal, healthy urinary proteome

Many factors not related to disease pathogenesis contribute variability to the urine proteome. Gender, age, diet, exercise, hormone status, diurnal variation, genetic variation in renal physiology and environmental factors create variability in the normal urine proteome [12, 13, 51, 109-113]. Which proteins emanate from plasma filtration versus renal secretion? How much do urinary proteins fluctuate over time in an individual? Are these proteins of pathologic significance? Can we establish expected, or ‘normal’, ranges for specific urinary proteins? The answer to these questions are a fundamental step in determining the “expected healthy urinary proteome” since normal ranges for urinary proteins have not been established except for a few high abundance, disease indicative proteins such as albumin, immunoglobulins, transferrin, and β-2-microglobulin [81]. The presence of “age dependent” urinary proteins has been associated with normal healthy states [51, 111] and diet and exercise affect the urinary proteome [10, 11, 113]. These findings indicate that large scale, longitudinal studies of individuals will be needed to establish a “healthy” living diagnostic urine profile.

Several studies have begun to address the questions posed above by characterizing urine proteins from healthy and diseased individuals [12, 13, 114-116]. To ascertain the source of urinary proteins, as plasma filtrates versus urogenital secretion, Jia *et. al.* compared publically available urine proteomic data sets, emanating from various institutions, methodologies, and individuals [8, 67, 117] with plasma proteome data sets (Human Proteome Organization Plasma Proteome Project and data from States *et. al.* [118]). The goal of this bioinformatics study was to characterize the proteins as plasma and/or urine sub-proteomes, which could potentially be used to enhance renal disease diagnostics and prognostics [3]. Gene Ontology (GO) terms statistically overrepresented in the plasma-and-urine sub-proteome versus the whole plasma proteome were searched using the Biological Networks Gene Ontology (BiNGO) program. Despite the complexities and potentially

confounding differences in data sets, Jia *et. al.* were able to classify the proteins based on GO terms into the plasma derived sub-proteome and the urine sub-proteome [3]. The plasma sub-proteome was comprised predominately of plasma proteins filtered by the glomerulus and soluble proteins secreted by epithelial cells. The urine-only sub-proteome stemmed mainly from soluble proteins secreted by epithelial cells [3].

To identify constituents of the typical “healthy” urinary proteome, multiplex bead immunoassays (xMAP, Luminex) have been utilized for profiling urine from apparently healthy individuals representing different ethnicities, genders, and tobacco use statuses (n=103) [115]. The multiplex analysis (211 proteins) included panels of cytokines, growth factors, and hormone receptors. Proteins were categorized based on relative abundance, after normalizing to urine creatinine concentrations: high 100ng/ml - >10ng/mL, average 10ng/ml - 100ng/mL, or low 1ng/ml - 10ng/mL abundance. Eight low molecular weight proteins were identified in the high abundance class. The average and low abundance classes exhibited diverse proteins. The average abundance proteins included proteases, soluble receptors, hormones, immunologic and growth factors [115]. The low abundance proteins consisted of cytokines, extra cellular matrix components, and matrix metalloproteinases [115]. Depending on the normalization method, the CV ranged from <10% for absolute values to 700% for urine creatinine normalized values [115]. Depending on the data normalization method, using urine creatinine, urine total protein, urine albumin, the ratio of urine albumin to urine creatinine, or β-2-microglobulin, the Pearson correlation coefficient ranged from 0.1765 for β-2-microglobulin normalized data, to 0.9457 when the data was normalized to urine total protein.

7. Data normalization issues for comparing urinary proteomes

A critical data normalization issue plaguing the urinary proteomics field was underscored in the healthy urinary proteome study [115]: What is the optimal parameter(s) to use for comparing urinary proteomic data within individuals over time, between individuals, or between laboratories and studies? Housekeeping genes, such as lactate dehydrogenase A (LDHA), have been used to normalize genomic data because LDHA is expressed at relatively constant levels and is tolerant to many, but not all, cellular perturbations [119]. To date, a single invariant urinary protein has not been identified that can be the “North Star” for guiding urinary protein normalization.

Although the correlation differences noted above in the healthy urinary proteome study accentuate some of the hurdles involved in defining/normalizing the urinary protein, Kentsis *et. al.* have identified a core set of “generally invariant proteins” in children (age range 1-18 years) that may serve as benchmark proteins for longitudinal evaluation of individuals [114]. Furthermore, non-protein analytes, such as creatinine [26, 45, 83, 115], neopterin [26, 120], or post-translationally modified proteins such as glycosylated hemoglobin (A1c) [121] can also be used to normalize urine proteomic data. Serum and urine creatinine levels are often used alone, or in combination with other parameters, to assess glomerular filtration because creatinine is completely filtered by the kidney and excreted in the urine [122]. For diverse patient populations with end-stage renal disease, Cystatin C alone or in combination with creatinine improved the association between the estimated Glomerular Filtration Rate

(eGFR) and the risks of death and end-stage renal disease [122]. In light of the pre-analytical variables and high inter-individual and intra-individual variation in urinary proteomes, it is essential for translational research studies to select case and control specimens that represent: a) the diseased population harboring the condition being studied, b) a diseased population not harboring the condition being studied, and c) healthy individuals. In addition, it may be necessary to combine multiple parameters from cellular, biochemical, and proteomic urine analytes to develop the optimal data normalization factor.

8. Enriching urine specimens for low abundance proteins

The work flow for urinary proteomics follows the general steps of sample collection, sample clarification, protein concentration/depletion/enrichment, and analysis (Figure 1). An analytical hurdle faced in proteomic analyses is the presence of high abundance proteins that can mask low abundance proteins [4, 15, 22, 23, 58, 123]. One approach to solve the problem of hidden, low abundance proteins is development of sensitive instruments, such as capillary electrophoresis time of flight mass spectrometry (CE-TOF-MS) [124], and the recently introduced capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) for analysis of amino acids in urine [125]. CE-MS data has been reported to be highly sensitive (<1 fmol) with 85% recovery from the sample preparation, high resolution of monoisotopic peaks with mass accuracy <25ppm, and <100ppm for unresolved peaks (z<6) [126]. Rather than inventing new, more sensitive instruments, alternative approaches for detecting low abundance proteins rely on devising technologies for subtracting interfering high abundance proteins, enriching the low abundance protein fraction, or equalizing the abundance of both high and low abundance proteins. Two techniques for enriching low abundance proteins highlighted in this review are combinatorial peptide ligand libraries to enrich the low abundance proteins (CPLL, ProteoMiner™) (Figure 2) [22, 24, 104, 123, 127-130] and hydrogel nanoparticles (NP) for excluding high molecular weight proteins and simultaneously harvesting specific classes of proteins (Figure 3) (Table 3) [23, 57, 107, 131, 132].

One means of enriching a sample for your proteins of interest is to deplete the unwanted proteins. Bulk methods including ultrafiltration and immunodepletion have been widely used to deplete high abundance proteins, for example albumin, transferrin, immunoglobulins, and haptoglobin. Ultrafiltration, also known as filter aided separation, uses column chromatography to eliminate proteins above a specific molecular weight, usually in the range of >10-30 kDa. The proteins retained in the column are washed with buffers to remove salts and chemicals. The column-retained proteins are digested and the resulting peptides are eluted from the column [103]. Ultrafiltration effectively concentrates large volumes of urine prior to mass spectrometry [88, 109, 116, 126, 133]. Precipitation of proteins via ice cold ethanol or trichloroacetic acid allows concentration of soluble urine proteins [12, 111, 134]. Specific classes of protein can be depleted using immunodepletion [102]. Filip *et.al.* evaluated commercially available immunodepletion columns (Seppro IgY 14, ProteoPrep and SpinTrap) and an ion-exchange column (ProteoSep) for their ability to enhance detection of low abundance urine proteins using LC-MS/MS [102]. Fractionated and unfractionated specimens from patients with chronic kidney disease and healthy patients were compared by 1-D gel electrophoresis and nano-LC QTOF mass spectrometry. The

immunodepletion columns were superior for depleting albumin fractions compared to the ion-exchange column. Fewer proteins were detected in the urine from chronic kidney disease patients, suggesting that the low abundance proteins were most likely masked by abundant urinary proteins that weren't depleted by the plasma protein depletion columns, or the mass spectrometer sensitivity was inadequate [102].

9. Combinatorial peptide ligand libraries

A second means of enriching a specimen for low abundance proteins is to selectively absorb proteins under capacity-limiting conditions [25]. Combinatorial peptide ligand libraries consist of hexamer peptides bound to a resin bead, with each bead containing millions of copies of a unique hexapeptide and a library of beads comprising many different hexapeptides on their respective beads [25]. This solid-phase hexapeptide library enriches low abundance proteins and also decreases the concentration of high abundance proteins, while representing all protein moieties in the original urine specimen [22]. CPLL were designed on the principles of affinity chromatography in which a chemical carrier is attached to a solid support to allow binding of a complimentary protein ligand. The physicochemical properties of a protein are determined by the amino acid sequence and their spatial configuration [25]. CPLL-ligand interactions vary from weak to strong depending on the type of physicochemical interaction (van der Waals interaction, hydrogen bonding, hydrophobic, ionic) [22]. Thus a mixture of many hexapeptide ligands could theoretically bind every protein in a complex milieu such as serum or urine. Each hexapeptide binds its respective protein ligand until saturation is reached, thus equalizing the concentration between low and high abundance proteins [130]. Increasing the volume of sample in relation to a fixed number of hexapeptide beads enhances the number of low abundance proteins [135]. 1M ammonium sulfate may be used to enhance hydrophobic hexapeptide-complementary protein interactions on CPLL [136]. Unbound/weakly bound proteins are commonly removed by washing, and the hexapeptide-bound proteins are eluted using appropriate conditions for the protein of interest/detection method (Figure 2). A potential limitation of using CPLL to enrich low abundance proteins is the low specificity of CPLL binding [135], however this issue has been addressed by standardizing the elution protocols [135], optimizing the elution schemes [7, 104, 128, 135], and performing pilot studies for different biological hypotheses.

Proteins bound to the CPLL may be directly trypsinized from the beads for direct LC-MS/MS analysis [137, 138]. One must use more trypsin than that used for in-gel digestion to allow the trypsin to reach all areas within the hexapeptide coated bead. Direct CPLL trypsinization ensures that even minute amounts of proteins are digested for analysis [137, 138]. A more common strategy is to eluate/desorb the bound proteins. Proteins can be eluted either globally from all beads at the same time, or specific classes of proteins may be eluted from the beads by selecting specific elution conditions (pH, chaotropic agent, ionic strength) [72]. Elution schemes for CPLL consist of a) disruption of hydrogen and ionic bond interactions with 100mM Tris, pH 7.4, 100mM lysine, pH 7.4, or a mixture of Arginine, Lysine, Histidine and Glutamine [104], b) disruption of all types of protein-ligand interactions with detergent and a reducing agent (2-4% sodium dodecyl sulfate and 2.5-3% dithiothreitol) [135], c) dissociation of ionic and electrostatic interactions with guanidine

hydrochloride [129], or d) disruption of hydrophobic interactions with urea-thiourea-cysteic acid [129]. Native proteins can be enriched in urine using the four amino acid mixture [104]. It should be noted that bile salts and urinary pigments (Urobilin, urochrome, uroerythrin) [139] present in urine were observed to bind strongly to CPLL, thus quenching albumin binding; however this same lack of albumin binding may not occur in kidney disease in which urinary pigment is decreased [104]. Urinary pigments were observed to be increased in patients in the third trimester of pregnancy, during high fever, Grave's Disease, and short periods of fasting [139]. Fortunately, butanol extraction can remove the interfering urine pigments [7, 129].

An optimized protocol for separation of urinary exosomes, utilizing CPLL to enrich low abundance proteins, and linear trap quadarople (LTQ) Orbitrap mass spectrometry has been developed by Santucci *et. al.* [7, 106]. Second morning urine specimens were stabilized with protease inhibitors on ice, clarified by centrifugation, and frozen. Following thawing, the specimens were spun at high speed, dialyzed against 25mM sodium phosphate, and refrozen. To isolate exosomes, supernatant from the high speed centrifugation was reduced with dithiothreitol to prevent exosome aggregation with uromodulin, followed by ultracentrifugation. The supernatant from the ultracentrifugation was dialyzed against water, acidified and precipitated with butanol. The butanol extraction phase was lyophilized for CPLL treatment [7]. Using this protocol, they detected 1724 new urinary proteins, many of which were found in exosomes [7].

The newest iteration of CPLL contain Alcian blue dye coupled to succinylated hexamers of hexapeptides to enhance protein binding [6]. By adding the copper containing phthalocyanine dye, Alcian blue, to the succinylated hexamers of CPLL, 115 urinary proteins with nucleotide, carbohydrate or cation binding, or catalytic activity were detected [6]. Combining CPLL equilibration techniques and physicochemical dye interactions into one capture moiety has revealed 38 previously undetected urine proteins, increasing the total urinary proteome to 4430 gene products [6]. By substituting the dye or altering the functional group of the hexapeptide libraries via different chemical transformations, a plethora of new protein harvesting agents can be synthesized to explore the urinary proteome.

10. Hydrogel nanoparticles

Hydrogel nanoparticles (NP) are three-dimensional hydrophilic copolymers of poly (N-isopropylacrylamide) (pNIPAm) and other compounds that change conformation from an extended coil to a globular structure in aqueous solutions [23, 140]. Hydrogel nanoparticles are synthesized via monomer polymerization with a cross-linking agent, which itself is a monomer with functional moieties that can also be polymerized [23]. These responsive hydrogels change solvation properties in response to temperature, pH, and ionic strength, providing added functionality to the polymer [23]. Hydrogel nanoparticles swell at lower temperatures, thus increasing their porosity at room temperature (500-700nm diameter) [23, 140]. This thermo-reversible property of hydrogel pNIPAm copolymers with acrylic acid has been extensively tested as a drug delivery vehicle [140].

Capitalizing on the NP modifiable functionality, Luchini *et. al.* synthesized hydrogel nanoparticles for molecular sieving, in solution, to sequester rather than release proteins [23, 107, 141]. The hydrogel nanoparticles can be easily modified by changing the polymer:cross linking agent ratio, thus controlling the nanoparticle size and effective porosity independently of temperature, while still maintaining the thermos-response properties of the hydrogel [23]. These characteristics allow the NP to rapidly harvest molecules out of solution due to the nanoparticle's large, tunable surface area, porosity, hydrophilicity, buoyancy, and dual hydrophobic and hydrophilic chemical moieties that can be substituted in the polymer [23, 107, 141]. In addition, NIPAm and acrylic acid copolymer nanoparticles were synthesized to incorporate an organic dye within the nanoparticle structure as a bait molecule for capturing low abundance proteins out of solution [23, 107, 141]. These common histology and affinity chromatography dyes have a high affinity for select classes of proteins based on the corresponding dye/protein physiochemical properties and hydrophobic interactions [23, 107, 141]. The nanoparticle pore size acts as a molecular sieve, effectively preventing large proteins such as albumin and immunoglobulins from entering the pores [107]. Another desirable property of the NP for harvesting low abundance proteins is their ability to protect their protein cargo from endogenous enzymatic digestion [23].

The ratio of nanoparticles to specimen volume can be adjusted to effectively capture 100% of the low abundance proteins in the specimen, hence amplifying the sensitivity of downstream analysis, including standard mass spectrometry, immunoassays, or western blotting [142]. In one step, in solution, hydrogel nanoparticles perform molecular size sieving, exclude high abundance proteins, capture classes of proteins based on the dye's functional group properties, and protect the captured proteins from enzymatic degradation [142]. Hydrogel nanoparticles are now commercially available in large quantities with uniform size distribution (NanoTrap ®) for harvesting and concentrating proteins in body fluids [143].

Hydrogel nanoparticle protein harvesting has been used to concentrate human growth hormone in serum and urine specimens, allowing the first estimate of growth hormone levels in human urine [132, 144, 145]. Growth hormone levels are important during clinical evaluation of growth hormone insufficiency. Growth hormone is secreted in pulses from the somatrotroph cells of the pituitary gland resulting in wide fluctuations in blood levels, with peak levels of 50-100ng/mL and less than 0.03ng/mL at baseline [144]. Pathophysiologic factors including gender, age, sleep, physical exertion, nutritional and metabolic factors, influence growth hormone secretion. The normal serum concentration of growth hormone is 1-10ng/mL, which is below the limit of detection of clinical grade immunoassays (50pg/mL) [144]. Growth hormone is efficiently filtered through the glomerulus, reabsorbed, and degraded in the proximal tubular cells; consequently the growth hormone concentration in urine is considerably less than in plasma [144]. Nanoparticles containing Cibacron Blue F3GA, a sulfonated triazine dye as the affinity bait, were used to harvest and concentrate urinary growth hormone from a healthy donor. The urine contained an estimated 0.175pg/mL of growth hormone, with 1.34 ng/mL in the donor matched serum specimen, thus confirming the ability of the hydrogel nanoparticles to concentrate very low abundance proteins [144].

Bacterial antigens can be harvested by hydrogel nanoparticles to enhance the sensitivity of western blot detection. Lyme Disease is transmitted via ticks infected with *Borrelia* spirochetes, commonly *B. burgdorferi* in the eastern United States. Diagnosis is typically made based on the presence of erythema migrans (bulls eye) rash and/or positive serologic test for antibodies to *Borrelia* antigens [57]. Lyme Disease can be difficult to diagnose in the early stages due to vague or overlapping symptoms, lack of the classic erythema migrans rash, or indeterminate serology results, thus potentially delaying treatment. As a proof-of-principle assay for developing a urine-based Lyme Disease assay, hydrogel nanoparticles were used to concentrate *B. burgdorferi* proteins spiked into urine [56]. NIPAm-acrylic acid nanoparticles containing the nondisperse dye Acid Black 48 were shown to sequester and concentrate *B. burgdorferi* outer surface proteins in urine, as well as from an individual *Ixodes scapularis* tick [56]. A non-invasive urine test to detect the presence of the *B. burgdorferi* antigens prior to development of antibodies could transform early diagnosis and treatment for Lyme Disease [57].

Following the proof-of-principle study, the dye bait was optimized for concentrating *Borrelia* antigens [57]. NP containing the anthraquinone dye Remazol Brilliant Blue were used to enhance the sensitivity of detection of *B. burgdorferi* via western blotting with a monoclonal antibody against a specific C-terminal OspA domain [57]. Urine specimens from 151 patients suspected of early stage or recurrent Lyme disease and 117 healthy controls were evaluated for the presence of urinary OspA [57]. Hydrogel nanoparticle harvesting of urinary proteins was able to confirm the presence of *Borrelia* OspA in untreated patients who had a characteristic erythema migrans rash. Furthermore, 41% of patients with suspected chronic Lyme *Borrellia* infection also were shown to harbor *Borrelia* OspA in their urine [57]. Clinical evaluation of the urine nanoparticle procedure is on-going.

Hydrogel nanoparticle protein harvesting has also been applied for diagnosing congenital transmission of Chagas Disease, caused by the parasite *Trypanosoma cruzi* [55]. Early detection of congenital Chagas Disease is difficult because of the low sensitivity of microscopic test and poor patient follow-up [55]. Using hydrogel nanoparticles containing the azo dye Trypan blue as the affinity bait, *T. cruzi* antigens were detectable in urine specimens by western blot with a monoclonal antibody against *T. cruzi* lipophosphoglycan, thus enabling an early diagnosis for congenital transmission of Chagas Disease [55]. The sensitivity of the NP enhanced western blot was 91.3%, with a specificity of 96.5% [55].

These aforementioned studies using combinatorial peptide libraries or hydrogel nanoparticle harvesting are heralding in a new era of urinary proteomics in which hormones, infectious disease antigens, and other very low abundance proteins and protein classes can be selectively enriched, identified, and quantified with existing immunoassays and mass spectrometers.

11. Urine proteome assays under development

Non-invasive assays for monitoring immediate blood glucose levels and long-term glucose control have the potential to dramatically impact the quality of life for diabetic patients. The non-invasive diagnostic trend encourages better diet/medication compliance and could

decrease health care costs. Recently, non-invasive methods for monitoring glucose levels using mid-infrared spectroscopy of interstitial fluid between the layers of skin have been developed [146]. Urine is also being explored as a surrogate to blood for monitoring diabetes-related biomarkers. Zhang *et. al.* applied matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and LC-MS/MS to urine specimens from patients with type 2 diabetes (n=49) and non-diabetic controls (n=49) to identify differentially expressed urinary proteins [121]. Urine proteins were concentrated using weak cation exchange chromatography with magnetic beads. A fragment of fibrinogen alpha chain precursor and a prothrombin precursor were upregulated in the diabetic patient specimens [121]. The authors speculate that the coagulation-associated peptides may reflect hypercoagulable states and impending diabetic vascular complications. Of note, the fibrinogen alpha chain precursor and prothrombin precursor were not statistically significant when the peptides were compared to blood glucose levels, but were significant when the data was compared to the glycosylated hemoglobin values [121]. Glycosylated hemoglobin (A1c) levels reflect long-term (over the course of 90 days) glucose control [147]. This difference in statistically significant data emphasizes the necessity of normalizing/comparing urinary proteomic data to a relevant biochemical assay in order to discern potentially relevant information.

Although glycosylated hemoglobin assays reflect long-term glucose levels, the standard assay uses whole blood collected by venipuncture [147]. We are developing a non-invasive urine assay based on hydrogel nanoparticle sequestration of A1c derived from urinary erythrocytes. The first step in developing this assay was to screen various dyes for the ability to capture glycosylated proteins, thus facilitating enrichment of A1c. We found a copper containing azo dye, Reactive Black 31 (RB31, CAS # 12731-63-4), to be the optimal chemical bait for capturing glycosylated hemoglobin in urine (Figure 4). The utility of RB31 dye was confirmed for protein harvesting by using synthetic urine (Surine) containing spiked-in serum. Compared to the starting solution without nanoparticles, the RB31 dye containing nanoparticles were able to concentrate proteins in this serum-Surine solution (Figure 5). The ultimate goal of a urine-based glycosylated hemoglobin assay is to develop a urine-based point-of-care test for measuring the ratio of A1c to total hemoglobin. Of course, this assay is in the preliminary stages of development and must undergo analytical validation for sensitivity, specificity, accuracy and precision as well as clinical validation for patients with diabetes [148].

12. Expert Commentary

Technological advancements in instrumentation and specimen processing for low abundance proteins are the catalysts driving the elucidation of the urinary proteome. Masking of low abundance proteins by high abundance proteins is not unique to urine specimens and fractionation techniques that have been developed for serum protein analysis are now being applied to urine [149].. These technological ehancements are providing insights into inter-individual and intra-individual variation of the urinary proteome. We are now glimpsing the reality of establishing normal values, or at least typical values, for urine proteins in certain populations [11, 113, 115].

Now that we can delve deeper into the body fluid proteome, we need to step back and reconsider the potential effects of specimen processing, i.e. the ‘spin first versus freeze first’ urine stabilization methods and fractionation methods, on the urinary proteome. This is particularly important when comparing studies for biomarker discovery in healthy versus disease states. Studies investigating pre-analytical variables are not typically considered newsworthy, but their importance impacts every clinical study seeking an answer to a biological question [28, 31, 35, 100, 101]. The main pre-analytical question that should be addressed for each urinary proteomic study is: Do the biochemical, uroepithelial cells, or bacterial cells present in the urine specimen contribute to the pathophysiology of the condition to be studied? Are these urinary proteins that we can now quantify derived from the plasma ultra-filtrate, or are they degradative products from the urogenital environment?

We shouldn't dismiss routine clinical biochemical and cytological urinalysis of urine specimens now that we can harvest, concentrate, identify, and quantify specific proteins/peptides. Urinalysis, including microscopic examination of the urine sediment, prior to proteomic biomarker discovery could reveal sources of false positive or false negative biomarkers [150]. Urine test strip (dipstick) assays are commercially available, rapid, and sensitive [27]. Furthermore, biochemical anlaytes measurable via a test strip, such as total protein, glucose, specific gravity, urobilinogen, glucose, ketones, blood, nitrite and leukocyte esterase, can potentially be used in data normalization and as specimen rejection criteria. Integration of clinical diagnostic assays with biomarker discovery will be the next leap in correlating urinary proteins/peptides with pathophysiology.

Urinary proteomic translational research has benefited greatly from dedicated clinicians and scientists who have recognized the need for: a) more studies that advance from initial testing in pilot studies, to controlled and validated studies, and finally progressing to large scale clinical trials, b) non-redundant urinary proteomic database access and maintenance, and c) adoption of clinical laboratory consensus statements and/or laboratory standards for comparability of results across analysis platforms and between laboratories [37, 151, 152]. These standards and guidelines facilitate discoveries by providing a framework for specimen collection, processing, analysis, and data reduction. Further innovations in urine specimen processing, exemplified by GlycoFilter sample preparation [97], CPLL-Alcian blue dye constructs [6], and hydrogel-dye nanoparticles [57], will reveal the true extent of urinary biomolecules and the plethora of molecular interactions waiting to be discovered, correlated, and integrated with treatment options for many different types of diseases/conditions.

13. Five-year View

Embracing new technology platforms and specimen processing methods to measure and detect the unseen or previously undetectable proteins, post-translational modifications, nucleic acids, exosomes, and infectious agents in urine will be a key driver in urinary proteomics. One size does not fit all, especially in clinical applications. Specimen processing procedures will need to be adjusted based on the clinical hypothesis, goal of the study, source of the molecule to be measured, the analytical platform, and/or the parameter for normalizing data.. Multi-omic biomarker panels may become more commonplace as biochemical, proteomic, genomic, and metabolomic tools and data are developed and

integrated into translational research studies to generate a molecular portrait for diagnosis, prognosis, and/or treatment efficacy.

We need to rethink how we are analyzing the urinary proteome. Rather than pooling specimens from multiple individuals, or from the same individual over time, we need to envision a future in which we can monitor/measure an individual patient's urinary proteome longitudinally. The individual patient would serve as their own baseline and we could monitor longitudinal fluctuations of inflammatory markers which are harbingers of cancer, infection, renal failure, etc. Improvements in point-of-care testing using mobile imaging such as with a cell phone camera [153, 154] are providing the technology to develop individual urine health monitoring devises. With miniaturized specimen processing modules and mobile imaging, we can envision ‘urine health trackers’ similar to the wearable wrist-style electronic health monitors.

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14. Key Issues

- The depth and breadth of the urinary proteome is increasing due to advances in analytical sensitivity and innovations in specimen processing to diminish high abundance urinary proteins that mask potential biomarkers within the low abundance proteome.
- Specimen processing impacts the content and quantity of urinary proteins/peptides.
- Specimen processing should be based on the clinical question being addressed in the study. Do the biochemicals, uroepithelial cells, or bacterial cells present in the urine specimen contribute to the pathophysiology of the condition to be studied? Are these urinary proteins that we can now quantify derived from the plasma ultra-filtrate, or are they degradative products from the urogenital environment?
- The urinary proteome exhibits inter and intra-individual variations. Population data from apparently healthy individuals is needed to develop normal ranges of urinary proteins/peptides.
- Pre-analytical variables need to be addressed in urinary proteome studies prior to starting the study.
- Urinary proteomics offers opportunities for development and validation of non-invasive diagnostics for monitoring immune function, as well as cardiovascular, renal, and infectious diseases.
- Further innovations in specimen processing will reveal the true extent of urinary biomolecules and the plethora of molecular interactions waiting to be discovered, correlated, and integrated with treatment options for many different types of diseases/conditions.
- Data normalization for urine proteomics presents challenges not encountered in serum/plasma proteomic studies because urine volume is highly variable between and within individuals, unlike the relatively stable blood volume. Urine proteomic studies will likely require a multi-analyte normalization method that incorporates excretion, secretion, and filtration parameters to be able to compare urinary proteomic data.

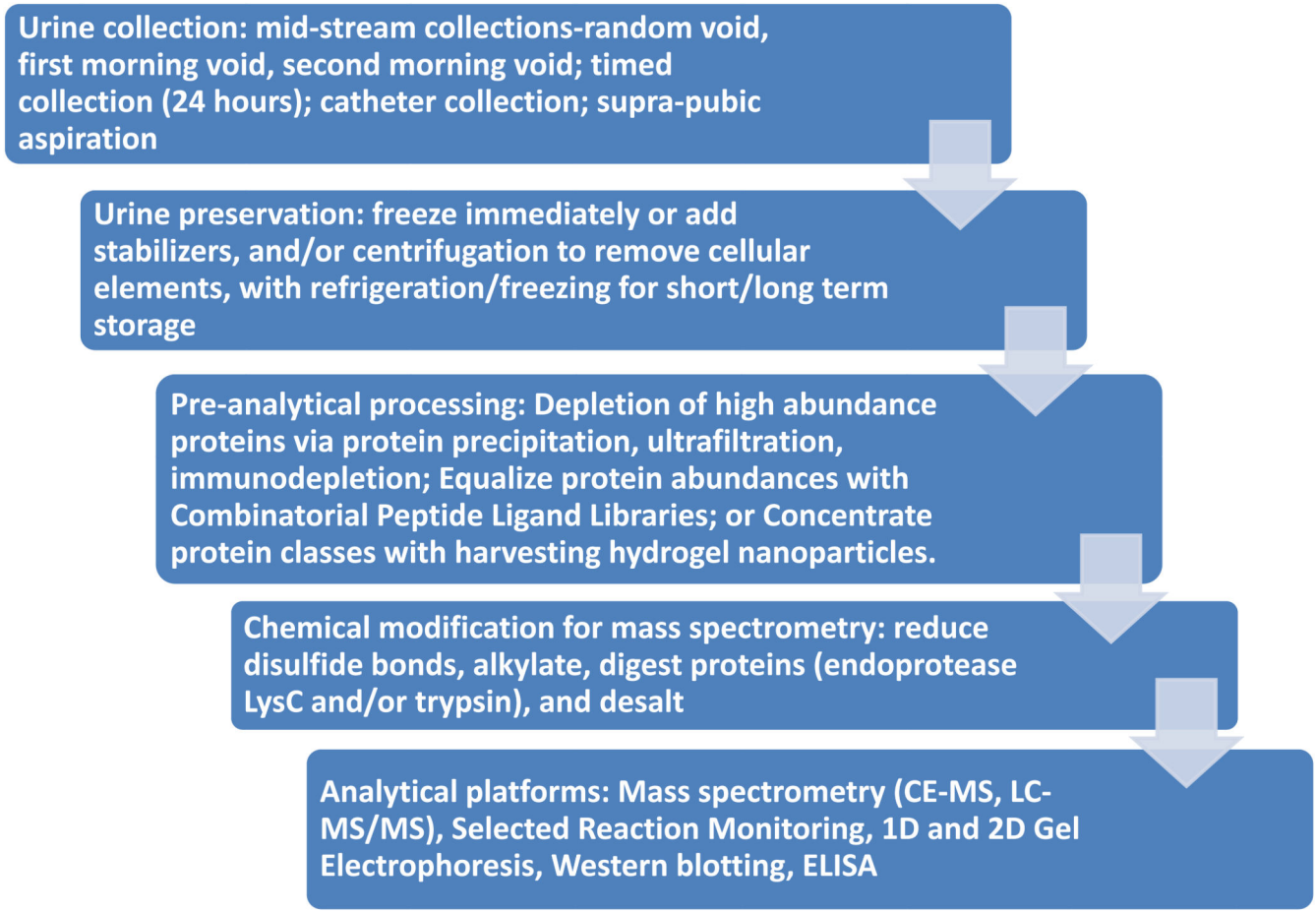

Figure 1. Example workflow for deciphering the urinary proteome/peptidome

Each step of the workflow should be optimized based on pathophysiology, and pre-analytical, analytical, post-analytical variables. Freezing urine specimens prior to centrifugation ensures that the cellular elements will be lysed and the cytoplasmic contents will contribute to the overall proteomic signature. Due to the large dynamic range of urinary protein concentrations, a variety of technologies are utilized to enhance the ability to detect low abundance urine proteins/peptides. Not all analytical platforms perform each step; however, the processes that are utilized in the test set/training set/pilot study should be identical to the processes used in the validation and verification studies.

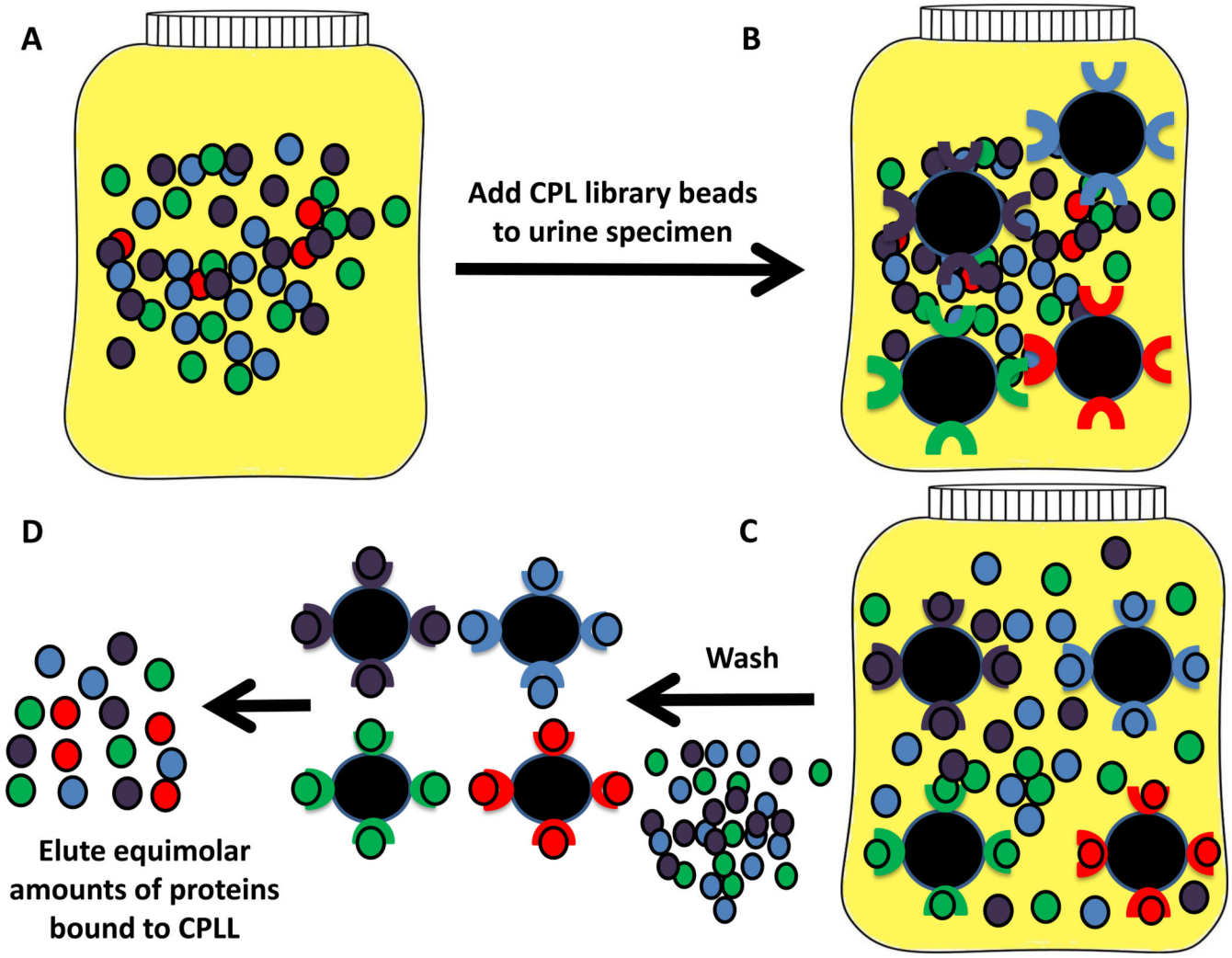

Figure 2. Urine biomarker discovery using combinatorial peptide ligand libraries (CPLL)

CPLL consist of random hexamers of amino acids bound to beads, with one type of hexamer per bead [6, 22, 25, 72]. This approach utilizes a saturation-depletion strategy wherein high abundance proteins will saturate their ligand first, leaving the excess to be washed off, while the low abundance proteins will be able to bind continuously. This strategy minimizes the concentration difference between the low and high abundance proteins. A. Initial urine solution, B. CPLL beads incubated with urine, C. Protein binding to the hexamer bound beads occurs via van der Waals interactions, ionic interaction, and hydrogen bonding. Non-adsorbed, high abundance proteins are removed via washing, D. The hexamer-bound proteins may be recovered en masse, or by protein class, based on standard chromatography elution conditions (pH, chaotropic agent, ionic strength).

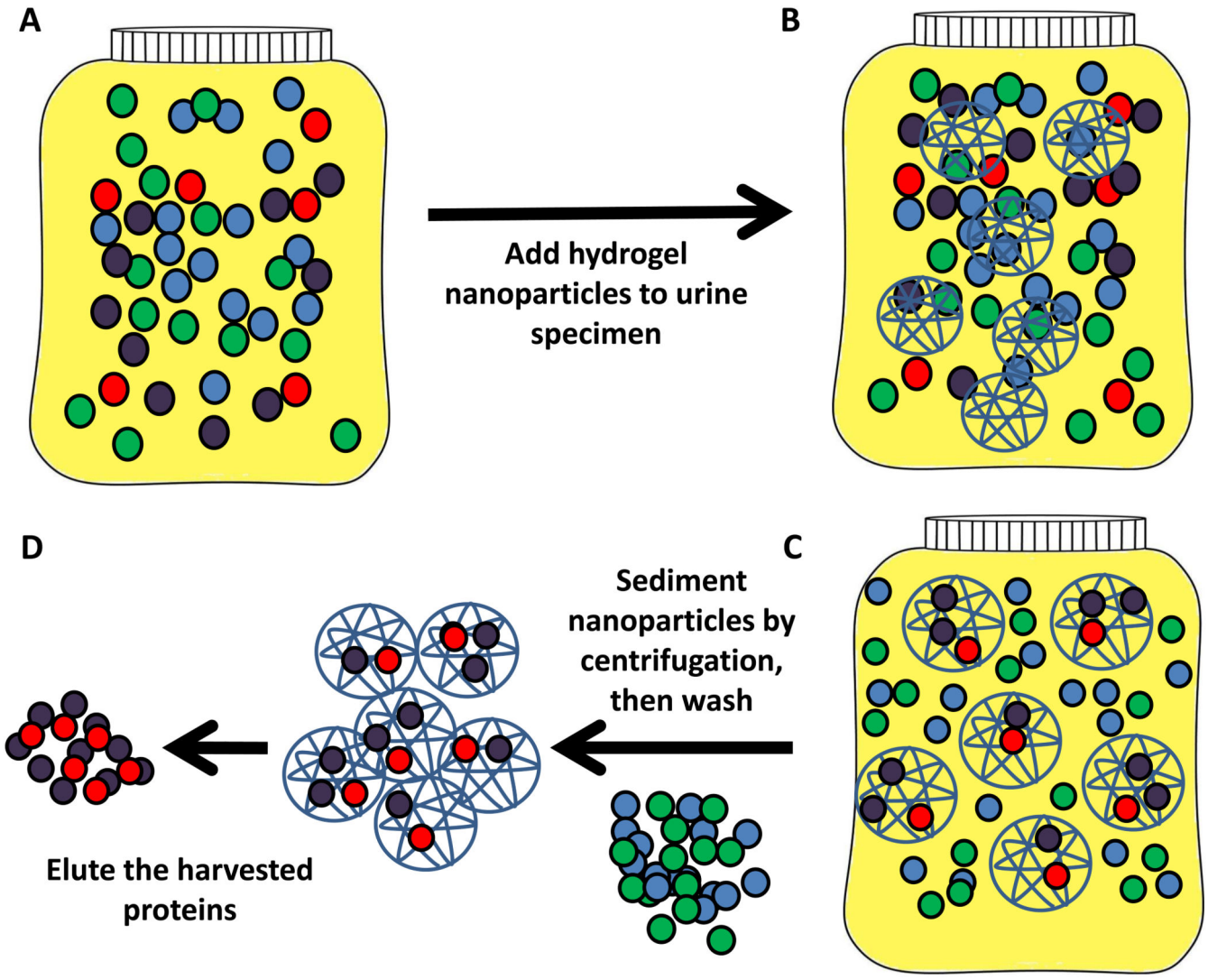

Figure 3. Protein enrichment strategy using hydrogel nanoparticles

Porous hydrogel nanoparticles contain a dye, which contains functional groups and/or metal ions, that enhance binding of specific classes of proteins. The porous hydrogel simultaneously excludes high molecular weight proteins such as albumin, while protecting the harvested proteins from endogenous enzymatic digestion [23, 57, 107]. The chemical bait can be selected to preferentially sequester classes of proteins, such as negatively charged or positively charged proteins. A. Urine containing two potential biomarkers (red and purple circles) and high abundance proteins (green and blue circles). B. Nanoparticle incubation with the urine specimen. C. Nanoparticles concentrate the low abundance proteins. D. Following centrifugation to sediment the nanoparticles, the excluded, high abundance proteins are washed off and the concentrated, low abundance proteins are eluted from the particles.

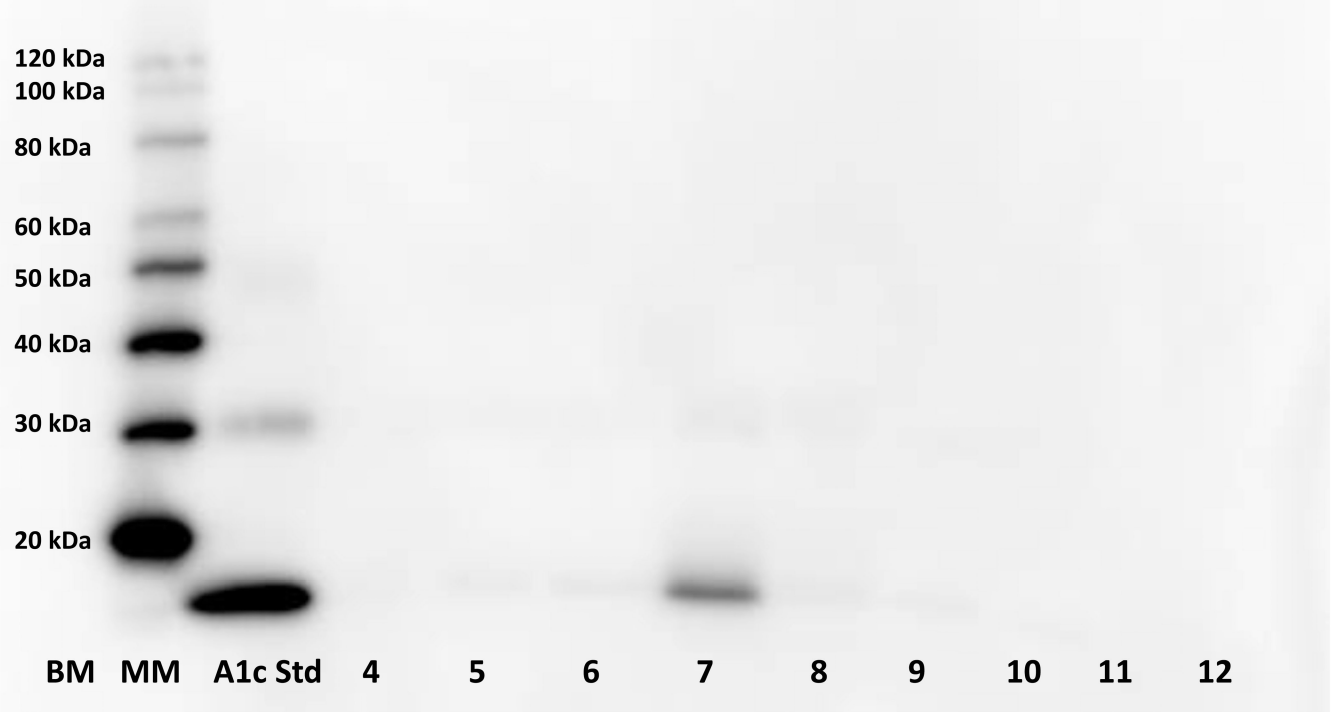

Figure 4. Proof of principle for developing a urine-based glycosylated hemoglobin (A1c) assay

Different dyes were incorporated into hydrogel nanoparticles to determine which nanoparticle bait (dye) was optimal for harvesting A1c. 100ng A1c (LEE Biosolutions cat#325-10, 15kD) was added to 5mL of Surine™ (synthetic urine negative control, Sigma-Aldrich) prior to adding the nanoparticles. Following elution from the nanoparticles, western blotting with anti-A1c (LifespanBio, LS-C194149-250) was used to demonstrate A1c capture by the nanoparticles. Reactive Black 31 (Lane 7) was found to be the optimal dye for harvesting A1c. Lane 1: BenchMark pre-stained protein ladder, Lane 2: Magic Mark chemiluminescent protein standard, Lane 3: 50ng A1c, Lane 4: Trypan Blue, Lane 5: Congo Red, Lane 6: Direct Blue 2, Lane 7: Reactive Black 31, Lane 8: Reactive Blue 221, Lane 9: Remazol Brilliant Blue, Lane 10: Sequential harvesting with Trypan Blue then Reactive Blue 221 nanoparticles, Lane 11: Surine™ sans nanoparticles, Lane 12: empty. (Lanes 4-10=proteins eluted from the respective nanoparticle).

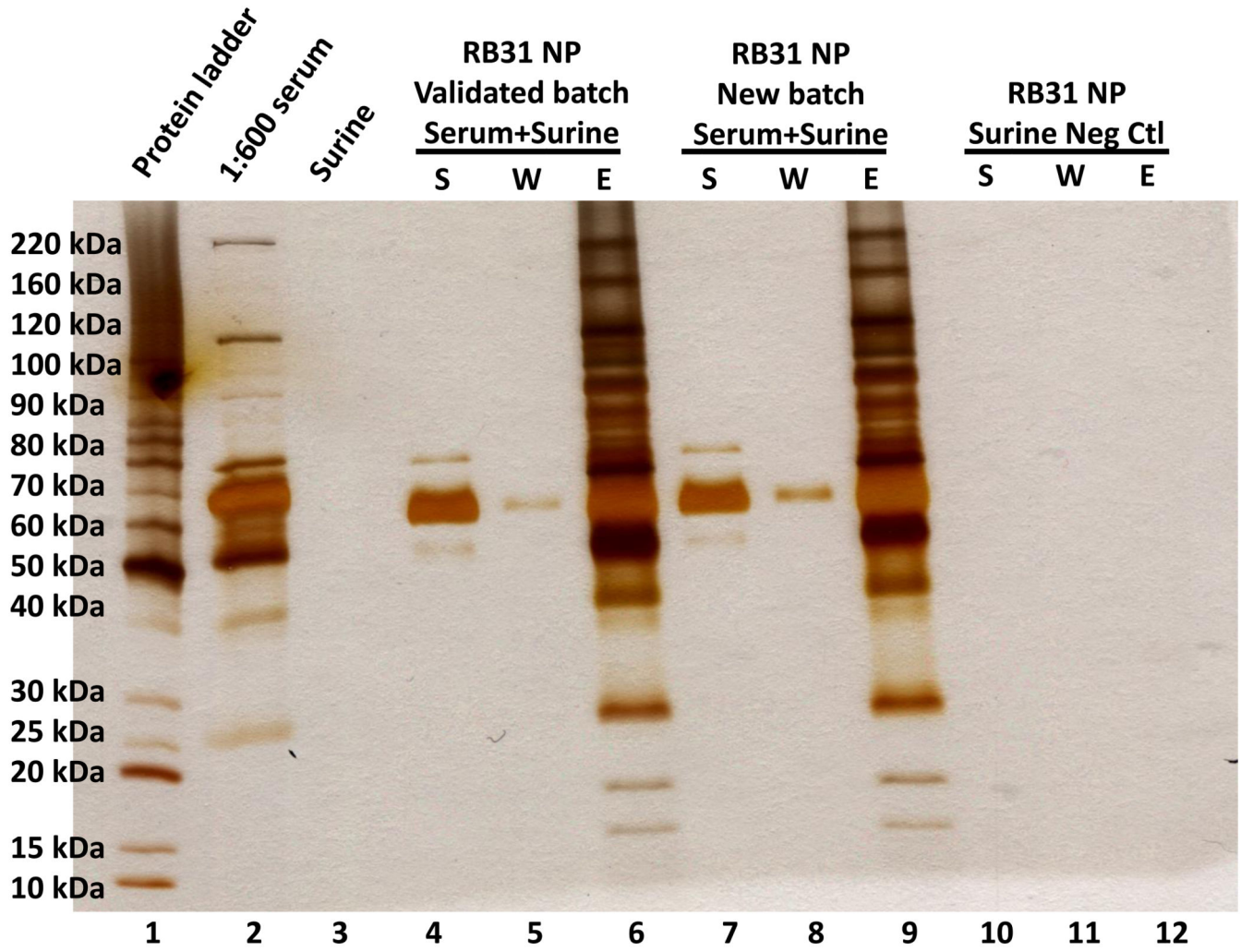

Figure 5. Validation of Reactive Black 31 nanoparticles (RB31 NP) for harvesting proteins

Serum (1:600 dilution) was spiked into synthetic urine (Surine™) to validate the protein harvesting ability of the Reactive Black 31 dye containing nanoparticles. The high abundance proteins remain in the supernatant, showing that they are not captured by the nanoparticles. A previously validated batch of nanoparticles was compared to a new batch and Surine™ (as a negative control). Lane 1: Benchmark unstained protein ladder, Lane 2: initial solution (1:600 serum), Lane 3: Surine™, Lane 4: supernatant of a previously validated RB31 NP batch, Lane 5: wash, Lane 6: elution, Lane 7: new batch of RB31 NP supernatant, Lane 8: new batch of RB31 NP wash, Lane 9: new batch of RB31 NP elution, Lane 10: supernatant negative control (Surine™), Lane 11: Surine™ wash, Lane 12: Surine™ elution.

Table 1

Advantages and disadvantages of urine specimens for protein biomarker discovery [1,2,28,30,31,44,89,155].

Parameter	Advantages	Disadvantages
Specimen volume	Large quantities available	
Ease of collection	Non-invasive collection	
Urine production	Urine is produced continuously throughout the day and can be collected to match normal diurnal variation. First morning urine samples are generally more concentrated than randomly collected specimens.	Some urinary proteins and chemical constituents are affected by diet, hydration, exercise, metabolism, circadian rhythms and hormones.
Protein quantity	Abundant source of soluble proteins/peptides that is less complex than blood.	
Protein stability	Urine proteins remain relatively stable at room temperature and 4°C for up to 6 hours.	
Pathophysiology	Urine reflects the plasma proteome as well as kidney proteome due to glomerular filtration, tubular reabsorption, and secretion.	A wide spectrum of renal pathologies produces similar urinary proteomic markers, *e.g.* albuminuria.
Constituents	Multiple chemical, cellular, and proteomic constituents are present in urine, many of which are well-defined in normal and pathologic states. Multiple analytes can be selected for data comparison between and within individuals/data sets.	Inter and intra-individual variability requires the use of multiple parameters for comparing urinary proteome data. Thoroughly annotated clinical information and/or urine biochemical and microscopic analyses may be needed to accurately compare urine proteomic data.
Analysis	Low molecular weight soluble proteins can be readily quantified by mass spectrometry. Urine proteins can be quantified by multiple methods. Urine biochemical analysis can be easily performed with urine test strips (dipstick). Cellular elements can be identified with bright field microscopy. Strategies for detecting low abundance urinary proteins are well documented and commercially available.	High abundance proteins mask the presence of low abundance proteins, thus requiring depletion and/or enrichment strategies for detecting low abundance proteins.

Table 2

Human kidney/bladder/urine proteome/peptidome resources for best practice guidelines, data repositories, and integrative knowledge databases.

Resource	Acronym	URL	Procedures/ Standards	Human Protein Data	Proteomic Methodologies	Reference
Chorus Project		https://chorusproject.org	n/a	User/experiment specific Cloud based platform for storage, analysis, and sharing of mass spectrometry data	Mass Spectrometry	[106]
Human Kidney & Urine Proteome Project	HKUPP	www.hkupp.org alos accessible from www.hupo.org	Urine exosome collection and processing	Glomerulus Renal medulla Urine-normal Urine-exosome	2-D Gel Electrophoresis CE-MS LC-MS/MS SELDI-TOF MS	[69,100]
Human Proteinpedia and Human Protein Reference Database	HPRD	http://www.humanproteinpedia.org/ http://www.hprd.org/	n/a	Portal for sharing/ integrating proteomic data	Mass Spectrometry Immunohistochemistry Immunofluorescence Western blotting Co-Immunoprecipitation Protein/Peptide microarray Yeast two hybrid screens	[70]
Human Proteome Organization	HUPO	www.hupo.org	Data interpretation guidelines	Links to multiple proteomic initiatives	Mass Spectrometry, antibodies, and the Knowledge Base	[159]
Human Protein Atlas	HPA	http://www.proteinatlas.org/	n/a	High-resolution images showing the spatial distribution of proteins in tissues	Immunohistochemistry Immunofluorescence RNASeq	[5,93,156,160,164]
Human Urine Database v3.0		http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front_content.php?idcat=257	n/a	25,000 peptides from independent samples for urine specimens from normal and diseased patients	CE-MS	[162]
Kidney & Urinary Pathway Knowledge Base	KUPKB	http://www.kupkb.org/#tab0	n/a	Information extracted from scientific publications and other related renal databases using Semantic Web search tools	Multiple proteomics data from published literature	[157] (note new URL after publication)
Max-Planck Unified Proteome Database	MAPU 2.0	http://www.mapuproteome.com/urine/	n/a	Protein sequences, function, motifs, references, & links to tissue sources and peptides	LTQ-FT and LTQ-Orbitrap mass spectrometer data	[67]
PeptideAtlas		www.peptideatlas.org/hupo/hkup	n/a	Data repository for annotation of eukaryotic	Mass Spectrometry	[42,71]

Resource	Acronym	URL	Procedures/ Standards	Human Protein Data	Proteomic Methodologies	Reference
				genomes via validation of expressed proteins		
ProteomeXchange		http://www.proteomexchange.org/	n/a	Provide a harmonized submission of MS proteomics data to the main existing repositories, and encourage data distribution	Shotgun mass spectrometry data sets	[163]
Sys-Body Fluid Database		http://www.biosino.org/bodyfluid/	n/a	11 body fluid databases compiled from scientific literature	Multiple data types	[158]
Urinary Exosomes Protein Database		https://hpcwebapps.cit.nih.gov/ESBL/Database/Exosome/	n/a	Urinary exosomes protein database with GO annotations	Mass Spectrometry	[68,69]
UniProt	UniProt	http://www.uniprot.org/	n/a	Unified view of protein sequence and functional information	Multiple data types	[165]
Urine Proteomics		Urineproteomics.org	n/a	Links to methods, databases, companies and organizations	Mass Spectrometry	[42,114]
Yale Protein Expression Database	YPED	http://yped.med.yale.edu/repository/Welcome.do;jsessionid=ECB816BF54EE909E9FFDC1F7A70F2551	n/a	Public access data repository	LCMS, MudPIT, ICAT, iTRAQ, SILAC, 2D Gel and DIGE, Progenesis and Skyline Label Free Quantitation, MRM analysis and SWATH	[89,161]

Table 3

Comparison of solid phase immunoadsorption, Combinatorial Peptide Ligand Libraries and hydrogel nanoparticles for separating urine proteins [22,23,25,105,107,129,166].

	Solid Phase immunoadsorption	**Combinatorial Peptide Ligand Libraries**	**Hydrogel nanoparticles**
Separation method	Subtraction	Equilibration enrichment	Enrichment/size exclusion
Protein interaction principles	Antigen-antibody interaction	Physicochemical interaction (including hydrophobic, van der Waals, hydrogen bonding) between complementary hexapeptides and proteins	Hydrophobic and electrostatic interactions between the dye bait and protein
Affinity molecule	Monoclonal or polyclonal antibody	Hexapeptides or hexapeptides +Alcian blue dye	Organic dyes Acrylic acid Vinylsulfonic acid
Size exclusion	No	No	Yes
Thermo-reversible	No	No	Yes
Low abundance protein enrichment	Possible	Yes	Yes
Affinity	Moderate (antibody dependent)	Weak-Strong	Strong
Specificity	High	Moderate	Moderate (dependent on the class of dye used as the affinity bait)