Video Article

Hydrogel Nanoparticle Harvesting of Plasma or Urine for Detecting Low Abundance Proteins

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Abstract

Novel biomarker discovery plays a crucial role in providing more sensitive and specific disease detection. Unfortunately many low-abundance biomarkers that exist in biological fluids cannot be easily detected with mass spectrometry or immunoassays because they are present in very low concentration, are labile, and are often masked by high-abundance proteins such as albumin or immunoglobulin. Bait containing poly(N-isopropylacrylamide) (NIPAm) based nanoparticles are able to overcome these physiological barriers. In one step they are able to capture, concentrate and preserve biomarkers from body fluids. Low-molecular weight analytes enter the core of the nanoparticle and are captured by different organic chemical dyes, which act as high affinity protein baits. The nanoparticles are able to concentrate the proteins of interest by several orders of magnitude. This concentration factor is sufficient to increase the protein level such that the proteins are within the detection limit of current mass spectrometers, western blotting, and immunoassays. Nanoparticles can be incubated with a plethora of biological fluids and they are able to greatly enrich the concentration of low-molecular weight proteins and peptides while excluding albumin and other high-molecular weight proteins. Our data show that a 10,000 fold amplification in the concentration of a particular analyte can be achieved, enabling mass spectrometry and immunoassays to detect previously undetectable biomarkers.

Video Link

The video component of this article can be found at http://www.jove.com/video/51789/

Introduction

Despite the completion of the human genome sequencing, significant progress has not been made in identifying biomarkers predictive of early stage disease, or that correlate with therapeutic outcome, or prognosis[1]. One reason for this lack of progress is that many potentially significant biomarkers exist at a concentration below the detection limit of conventional mass spectrometry and other biomarker discovery platforms. Mass spectrometry (MS) and Multiple Reaction Monitoring (MRM) have a detection sensitivity typically greater than 50 ng/ml while the majority of the analytes measured by immunoassays in a clinical laboratory fall in the range between 50 pg/ml and 10 ng/ml. This means that many biomarkers, particularly in the early stage of a disease cannot be detected by conventional MS and MRM[2]. In addition the presence of high-abundance proteins such as albumin and immunoglobulin in complex biological fluids often mask by billion-fold excess low-abundance, low molecular weight proteins and peptides[3, 4]. For this reason several sample preparatory steps are required prior to mass spectrometry sequencing and identification. One such preparatory step employs the depletion of high-abundance proteins with commercially available depletion columns[5-8]. Unfortunately this step leads to the reduction of the yield of candidate biomarkers because they are often non-covalently associated with carrier proteins that are being removed. Another challenge is represented by the stability of candidate biomarkers *ex-vivo* once the samples are collected. Proteins are subject to degradation by endogenous or exogenous proteases[9]. Hydrogel nanoparticles can transcend these critical challenges by amplifying the putative biomarker concentration to a level within the range of the assay, while protecting the protein from degradation[10-13].

It's important to note that LMW proteins in blood are a mixture of small intact proteins as well as fragments of large proteins. Tissue-derived proteins larger than 60 kDa are too large to passively enter the blood stream through the vascular basement membrane, but they can be represented in blood as peptides or protein fragments[14]. Our goal is to measure novel circulating biomarkers that can be candidates for early detection of disease, patient stratification for therapy, and monitoring the response to therapy. Our nanoparticles are created to selectively exclude high abundance immunoglobulins and albumin, while simultaneously capturing smaller proteins and peptides and concentrating them up to 100-fold depending on the starting volume.

Our group identified a set of small organic dyes which can successfully act as high affinity molecular baits for proteins and peptides. Protein-dye binding is thought to be due to a combination of hydrophobic and electrostatic interactions. The aromatic rings on the dye interleave with proteins via hydrophobic pockets on the protein surface[11].

The baits, depending on their chemistry, show a particular affinity for selected classes of analytes. The baits compete with the carrier proteins, such as albumin, for the proteins or peptides. The low-molecular weight proteins/peptides become trapped in the particle. High-molecular weight proteins such as albumin and immunoglobulin are prevented from entering the particle because of the sieving capability due to the restrictive pore of the hydrogel[11] **(Figure 1)**.

Hydrogel nanoparticles are synthesized by precipitation polymerization initiated by ammonium persulfate[11]. N-isopropylacrylamide (NIPAm), co-monomers of acrylic acid (AAc) and allylamine (AA) and cross-linker N,N'-Methylenebisacrylamide (BIS) are allowed to react at 70 °C for 6 hr in dilute conditions[11, 13]. The high protein binding affinity of poly(N-isopropylacrylamide-co-acrylic acid) (poly(NIPAm-co-AAc) nanoparticlesis achieved by covalently incorporating amino-containing dyes (*i.e.*, sulfonatedanthraquinonetriazine dyes) into the nanoparticles through an amidation reaction performed in aqueous or organic solvents depending on the hydrophilic/hydrophobic characteristics of the dyes[11, 13]. Nucleophilic substitution of the amine groups in the nanoparticle with the chloride atom of an anthraquinonetriazine dye is utilized to create dye-containing poly(NIPAm-co-Allylamine) (AA) nanoparticles[11, 12]. A two-step polymerization process is utilized to create hydrogel nanoparticles containing an outer shell of vinylsulfonic acid (VSA)[11, 13].

Hydrogel nanoparticles can be applied to various biological fluids, including whole blood, plasma, serum, cerebrospinal fluid, sweat, and urine. In one step, in solution, the nanoparticles perform a rapid (within minutes) sequestration and concentration of low molecular weight analytes[10, 11, 13, 15-18]. Proteins are subsequently eluted from the nanoparticles and detected using western blotting[19-21], mass spectrometry[10, 11, 13, 15, 18, 22, 23], immunoassays/ELISA[10, 11, 15, 18], or reverse phase protein microarray[16, 24] assays. Nanoparticles functionalized with chemical bait, and presenting a core or core shell architecture, capture and concentrate proteins based on the bait/shell physicochemical properties. Different dyes incorporated into the nanoparticles will therefore capture different subsets of proteins with varying efficiency based on the dye affinity, pH of the solution, and the presence/absence of competing high-abundant proteins[13]. Furthermore, the quantity of nanoparticles in relation to the volume of the solution will affect the protein yield from the nanoparticles. These aspects of hydrogel nanoparticle harvesting are demonstrated using three different nanoparticle baits for harvesting proteins from plasma samples which contain high amounts of protein, and from urine samples which typically do not contain large amounts of protein. In this protocol we demonstrate harvesting and concentrating tumor necrosis factor alpha (TNFα) from plasma samples using poly(NIPAm-co-AAc), Poly(NIPAm/dye), and core- shell nanoparticles (Poly(NIPAm-co-VSA)). Poly(NIPAm/dye) nanoparticles are shown to concentrate *Mycobacterium* species antigen that was added to human urine samples, to mimic *Mycobacterium tuberculosis* infected individuals.

Protocol

Human plasma and urine was collected from healthy volunteer donors, with written informed consent, following George Mason University Institutional Review Board approved protocols. Donors were equally distributed between Caucasian males and females between the ages of 25 and 42. Samples were analyzed individually and were not pooled.

1. Nanoparticle Processing of Serum or Plasma Samples

Potential low abundant biomarkers in plasma are captured, in solution, with hydrogel nanoparticles. The particles are added to the plasma, incubated, separated by centrifugation, washed, and the captured proteins are eluted. The eluted proteins are dried under nitrogen flow for downstream mass spectrometry sequencing and identification.

1. Dilute 500 µl of serum 1:2 with 50 mM TrisHCl pH 7 (500 µl serum + 500 µl TrisHCl) in a microcentrifuge tube.
2. Add 500 µL of poly(NIPAm/AAc) core nanoparticles. Incubate for 15 min at RT.
3. Spin the sample at 16,100 x g, 25 °C for 10 min in a centrifuge equipped with a fixed-angle rotor. Remove and discard the supernatant.
4. Add 500 µl sodium thiocyanate (25 mM) to the pellet. Resuspend the nanoparticles by vigorously pipetting up and down multiple times.
5. Spin the sample at 16,100 x g, 25 °C for 10 min in a centrifuge with a fixed angle rotor. Remove and discard the supernatant.
6. Add 500 µl of MilliQ water to the nanoparticle pellet. Resuspend the nanoparticles by vigorously pipetting up and down multiple times.
7. Spin the sample at 16,100 x g, 25 °C for 10 min in a centrifuge with a fixed angle rotor. Remove and discard the supernatant.
8. Prepare fresh elution buffer: Add 700 µl acetonitrile to 300 µl ammonium hydroxide in a clean tube. Caution: Ammonium hydroxide is corrosive. Use with appropriate ventilation. Note: The elution buffer must be prepared immediately before use. Do not store the elution buffer.
9. Add 300 µl of elution buffer to the nanoparticle pellet. Resuspend the nanoparticles by vigorously pipetting up and down multiple times. Incubate for 15 min at RT.
10. Spin the sample at 16,100 x g, 25 °C for 10 min in a centrifuge with a fixed angle rotor. Remove and save the eluate in a clean, labeled microcentrifuge tube.
11. Repeat steps 1.9 – 1.10. Combine the two eluates into one microcentrifuge tube.
12. Dry the eluates under nitrogen flow in a nitrogen evaporator manifold at 42.1 °C, with air flow set to 8 (**Figure 2F**).
13. Store the dried eluate at RT for O/N storage, or at -20 °C for long term storage, prior to mass spectrometry, western blotting, or ELISA assays (optional).

2. Nanoparticle Processing of Urine Samples

Normal urine contains less than 30 mg/dl protein and less than 1+ blood. However, many diseases/conditions may alter the normal levels of urine protein and blood. To aid in determining the optimal volume of nanoparticles to add to the urine sample, a urinalysis is performed prior to nanoparticle harvesting. Urine biomarkers may exist in extremely low concentrations, which may require optimizing the ratio of nanoparticles to urine volume. This procedure describes nanoparticle harvesting of urine samples for downstream western blot analysis.

1. Collect urine samples in a clean, dry plastic cup. A 22 ml minimum volume is required. Store urine specimens at -80 °C until ready to analyze.
2. Thaw the frozen urine at RT or at 4 °C O/N. Mix briefly on a vortex mixer. Pour at least 22 ml of urine into a 50 ml conical bottom polypropylene tube.

3. Spin the urine in a centrifuge with a swing-out rotor at 3,700 x g for 15 min. Decant the urine, without disturbing the pellet, into a clean 50 ml conical bottom polypropylene tube. Discard the pellet.
4. Perform urinalysis using a multi-analyte “urine dipstick” reagent strip. Note: store the reagent strips in a tightly closed container away from direct sunlight and humidity[17, 18].
    1. Lay the reagent strip, face-up, on a clean, dry paper towel.
    2. Use a disposable pipette to aspirate 1 ml of the urine prepared in step 2.3.
    3. Set a timer for 2 min but do not start it. Quickly dispense 1 - 2 drops of urine on each test pad of the reagent strip. Immediately start the timer. Note: Do not submerge the reagent strip directly in the urine container. The dye/chemical indicators in the reagent strip can potentially leach out of the reagent strip into the urine container.
    4. At the time indicated on the reagent strip container, record the qualitative results for the various analytes on the reagent strip by comparing the color of the individual reagent strips to the corresponding color-coded indicators on the reagent container. Typical normal urine results are: (normal or negative) for blood, protein, leukocytes, nitrite, glucose, ketone, bilirubin, and urobilinogen; Urine pH (5.5 - 7.0); specific gravity (1.001 - 1.020). Note: High protein levels in a urine specimen may compete with the protein of interest for binding sites on the nanoparticles. To maximize protein harvesting with nanoparticles, the nanoparticle volume/urine volume ratio can be adjusted to either limit competition from high abundance proteins, or can be optimized to harvest proteins that exist in very low concentrations. If the urine protein value is 1+ or greater, add 2x volumes of nanoparticles in step 2.5 below. If the analyte of interest is a very low abundant protein, it may be necessary to increase the volume of nanoparticles up to 2 ml **(Figure 3)**.

5. Transfer 20 ml of the clarified urine from step 2.3 into a clean 50 ml conical bottom polypropylene tube. Do not disturb any debris/pellet that may be in the bottom of the urine tube. Add 200 µl of nanoparticles to the 20 ml urine sample. Mix briefly on a vortex mixer. Note: If the urine protein value is 1+ or greater, add 400 µl of nanoparticles to 20 ml urine.
6. Incubate the urine/nanoparticle mixture for 30 min at RT, without rocking/mixing.
7. Spin the urine/nanoparticle suspension in a centrifuge equipped with a swing-out rotor at 3,700 x g for 10 min. Note: If a pellet is not visible following centrifugation, spin the samples for an additional 2 – 7 min.
8. Remove and discard the supernatant. Add 500 µl MilliQ water to the nanoparticle pellet.
9. Resuspend the nanoparticles by vigorously pipetting up and down multiple times. Transfer the nanoparticle solution to a clean 1.5 ml microcentrifuge tube.
10. Spin the nanoparticles in a centrifuge equipped with a fixed-angle rotor at 16,100 x g for 10 min. Note: If a pellet is not visible following centrifugation, spin the samples for an additional 2 – 7 min.
11. Remove and discard the supernatant.
12. Repeat steps 2.8 and 2.11 (twice) with 200 µl 18 MilliQ water to wash the nanoparticles.
13. Prepare fresh elution buffer: Add 970 µl acetonitrile to 30 µl ammonium hydroxide in a clean tube. Caution: Ammonium hydroxide is corrosive. Use with appropriate ventilation. Note: The elution buffer must be prepared immediately before use. Do not store the elution buffer.
14. Add 20 µl elution buffer to the nanoparticle pellet. Resuspend the nanoparticles by vigorously pipetting up and down multiple times. Incubate for 15 min at RT.
15. Spin the nanoparticle samples at 16,100 x g for 10 min. Remove and save the supernatant in a clean, 1.5 ml microcentrifuge tube. Do not disrupt the nanoparticle pellet. Discard the pellet in the biohazard trash.
16. Place the samples in a rack in a chemical fume hood. Open the caps and incubate at RT for 30 min. Alternatively, place the samples under nitrogen flow at 40 °C until dry (1 - 2 hr).
17. Add 15 µl Tris-glycine SDS sample buffer (2x) to the samples. Heat at 100 °C in a dry heat block for 5 min with the caps open or until the volume left in the tube is no more than 20 µl. Note: do not use a boiling water bath. The humidity from the water bath will prevent evaporation of the buffer.
18. Place the cap on the tubes. Remove the tubes from the heat block. The sample can be stored at -80 °C or used immediately for downstream western blot analysis.

Representative Results

Hydrogel Nanoparticle Size and Uniformity

Poly(NIPAm-AAc) particles have been produced with extremely high yield and reproducibility between and within batches. The particles have very good colloidal stability at RT during the time required for capture, storage, and elution of proteins (at least 48 hr), and nanoparticle precipitation has not been observed (**Figure 1**)[11]. The colloidal stability may be very important for rapid protein/peptide uptake by the nanoparticles.

Potential Low-abundance Biomarkers Harvested from Plasma

The incorporation of the dye bait drives the uptake of molecules in solution and assures that captured molecules are preserved from degradation[13, 15, 18, 25]. For this reason, protease inhibitors are not needed during sample pre-processing. This attribute of the nanoparticles makes them suitable as a preservation technology for sample collection in the field and for shipment of biological fluids at RT.

The bait molecule can be incorporated in the hydrogel particle by copolymerization or covalent binding to functional groups present in the nanoparticle. As an example, poly(NIPAm-co-AAc) nanoparticles were constructed with amino containing dyes via zero length crosslinking amidation reactions, while nanoparticles with an outer shell containing vinylsulfonic acid (VSA) copolymer were created by a second polymerization reaction[10-13]. Different types of nanoparticles can be produced by incorporating a different chemical dye within the nanoparticle and thus enhance harvesting of specific classes of proteins. An important concept of our technology is that different baits can show a preferential affinity for different proteins because the dye-protein interaction is dependent mainly on a combination of hydrophobic interactions, 3-D interactions, and electrostatic forces. We have observed that some analytes can be captured with a high affinity by chemically different dyes because of the complexity of the binding forces. See Tamburro *et al.* for an extensive explanation and examples of this principle[13]. For example, four different types of capture bait (nanoparticles) were used to harvest TNFα from plasma using 200 µl of particles and 200 µl plasma for each type of nanoparticle. In all cases after treatment with the nanoparticles, TNFα was not detectable in the supernatant by SDS-PAGE with silver

staining, whereas TNFα was detectable in the nanoparticle eluate (**Figure 1C**). (Lane 1 = recombinant TNFα (MW 17,000 Da), Lanes 2 & 3, 4 & 5, 6 & 7 and 8 & 9 represent results for four different types of nanoparticle baits respectively; C = control, S = supernatant, P = nanoparticle eluate).

Dose-response for Different Types of N anoparticles

In order to perform a calibration curve, and thus assess yield and precision, experiments need to be performed with spiked-in proteins of known concentration. Published results for a variety of body fluids and analytes demonstrate how the nanoparticle pre-processing maintains linearity of the assay and establishes a calibration curve while enhancing the effective limit of detection[10, 16, 26]. We have also published studies of between-laboratory precision using the nanoparticles to conduct multi-reaction monitoring mass spectrometry with enhanced sensitivity[27].

To compare IL-17 harvesting efficiency by different types of nanoparticle, recombinant IL-17 (17.5 kDa) was added to plasma samples to which either poly(NIPAm-co-AAc) or poly(NIPAm-co-VSA) nanoparticles were subsequently added. Two sets of four serial dilutions of 100 ng of recombinant IL-17 were prepared in 100 µl plasma (100 ng/100 µl, 50 ng/100 µl, 25 ng/100 µl and 12.5 ng/100 µl). poly(NIPAm-co-AAc) or poly(NIPAm-co-VSA) particles were added to the respective plasma samples in a 1:1 (v/v) particle:plasma ratio. A spectrophotometric total protein assay was performed on the supernatant samples. Western blotting with anti-rabbit polyclonal IL-17 was performed on 20 µg of plasma supernatant (S) after nanoparticle harvesting and the nanoparticle eluates (P) (**Figure 4**). Both nanoparticle types adequately harvested and concentrated IL-17 at each dilution. The additional bands on the western blot represent human serum albumin and immunoglobulin that cross-react with the secondary antibody. (AAc particles: Lanes 1 & 2 = 1 ng/µl IL-17, Lanes 3 & 4 = 0.50 ng/µl, Lanes 5 & 6 = 0.25 ng/µl, Lanes 7 & 8 = 0.125 ng/µl, Lane 9 = IL-17; VSA particles: Lanes 1 & 2 = 1 ng/µl IL-17, Lanes 3 & 4 = 0.50 ng/µl, Lanes 5 & 6 = 0.25 ng/µl, Lanes 7 & 8 = 0.125 ng/µl).

Detection of Potentially Diagnostic Proteins in Urine

Human urine samples, obtained from healthy volunteer donors with written informed consent, were spiked with a recombinant *Mycobacterium* species antigen Early Secretory Target Mycobacterium Tuberculosis protein (ESAT-6) to mimic *Mycobacterium tuberculosis* infected individuals. 1 µg each of ESAT-6 (15 kDa) and IL-2 (15.5 kDa) were added to 1 ml aliquots of human urine collected from healthy volunteer donors. Urinalysis was performed on the samples with a urine reagent strip to determine the optimal ratio of nanoparticles to urine, which was based on the presence/absence of urinary proteins. Poly(NIPAm/dye) nanoparticles were used to harvest proteins from treated and untreated urine samples. One-dimensional gel electrophoresis of the nanoparticle eluates was performed followed by silver staining. Samples in U4 and U6 lanes represent nanoparticle eluates from ESAT-6 and IL-2 treated urine samples, clearly showing prominent bands at 15 kDa (**Figure 5**). Mass spectrometry of the eluates identified ESAT-6 (UniProt accession POA567, 84.0 coverage, 9 peptides) and IL-2 (UniProt accession P60568, 38.56 coverage, 3 peptides). Urine without nanoparticle harvesting is shown in the lane labeled “Raw”.

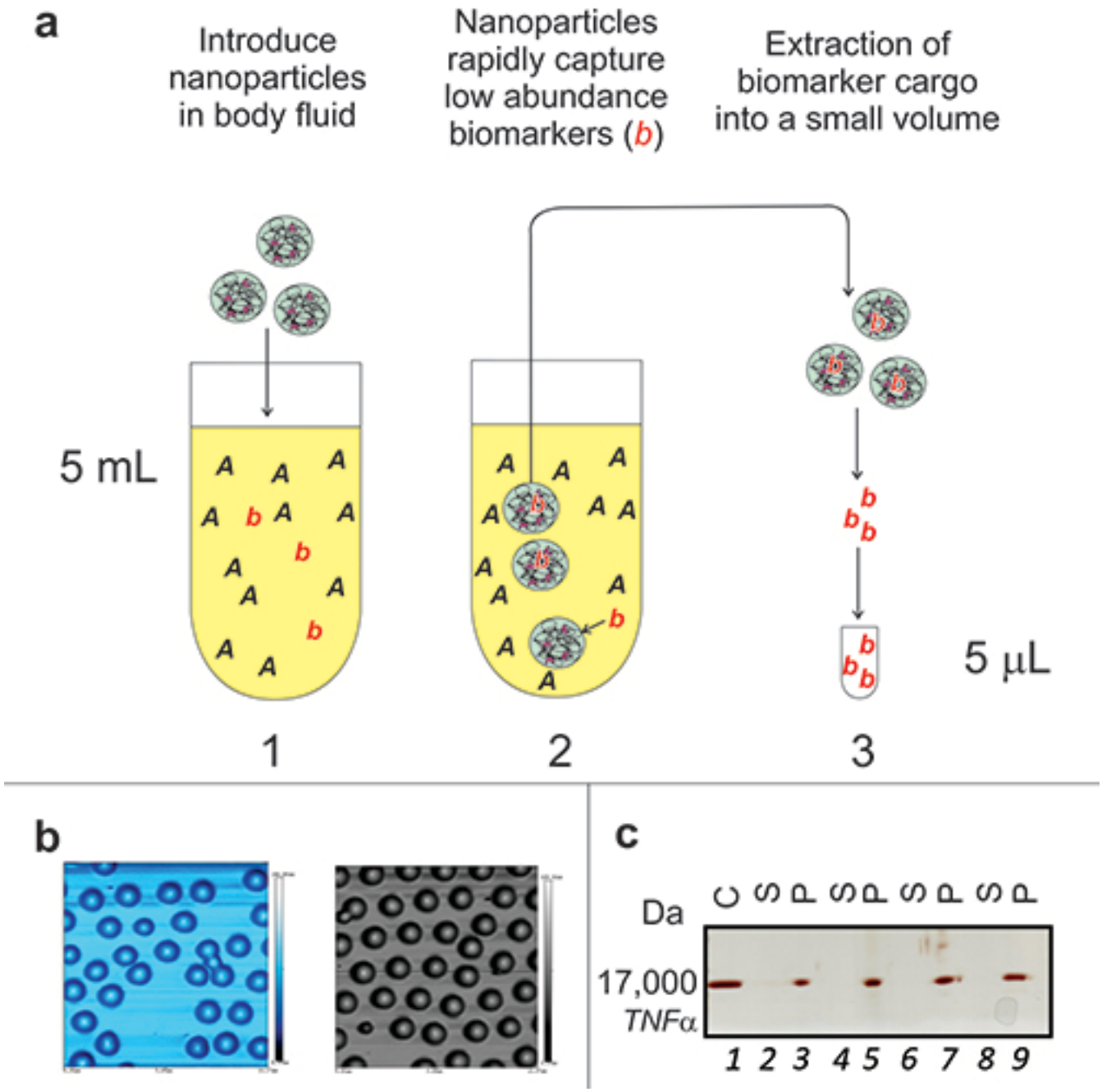

Figure 1. Nanoparticles harvest and concentrate low abundance proteins from complex biological fluids. (A) Workflow for harvesting proteins. Total processing time is approximately 1.5 hr. Proteins in solution are concentrated from blood, serum, plasma, urine, sweat, saliva, or other body fluids (1,000-fold concentration depicted). **(B)** Batch to batch comparison showing the uniform size of nanoparticles. Atomic Force Microscopy on mica shows uniformity of size (0.7 µm diameter) and absence of clumping in two batches of nanoparticles. **(C)** 1-D gel electrophoresis, with subsequent silver staining, of Tumor Necrosis Factor alpha (TNFα) sequestered from plasma. Please click here to view a larger version of this figure.

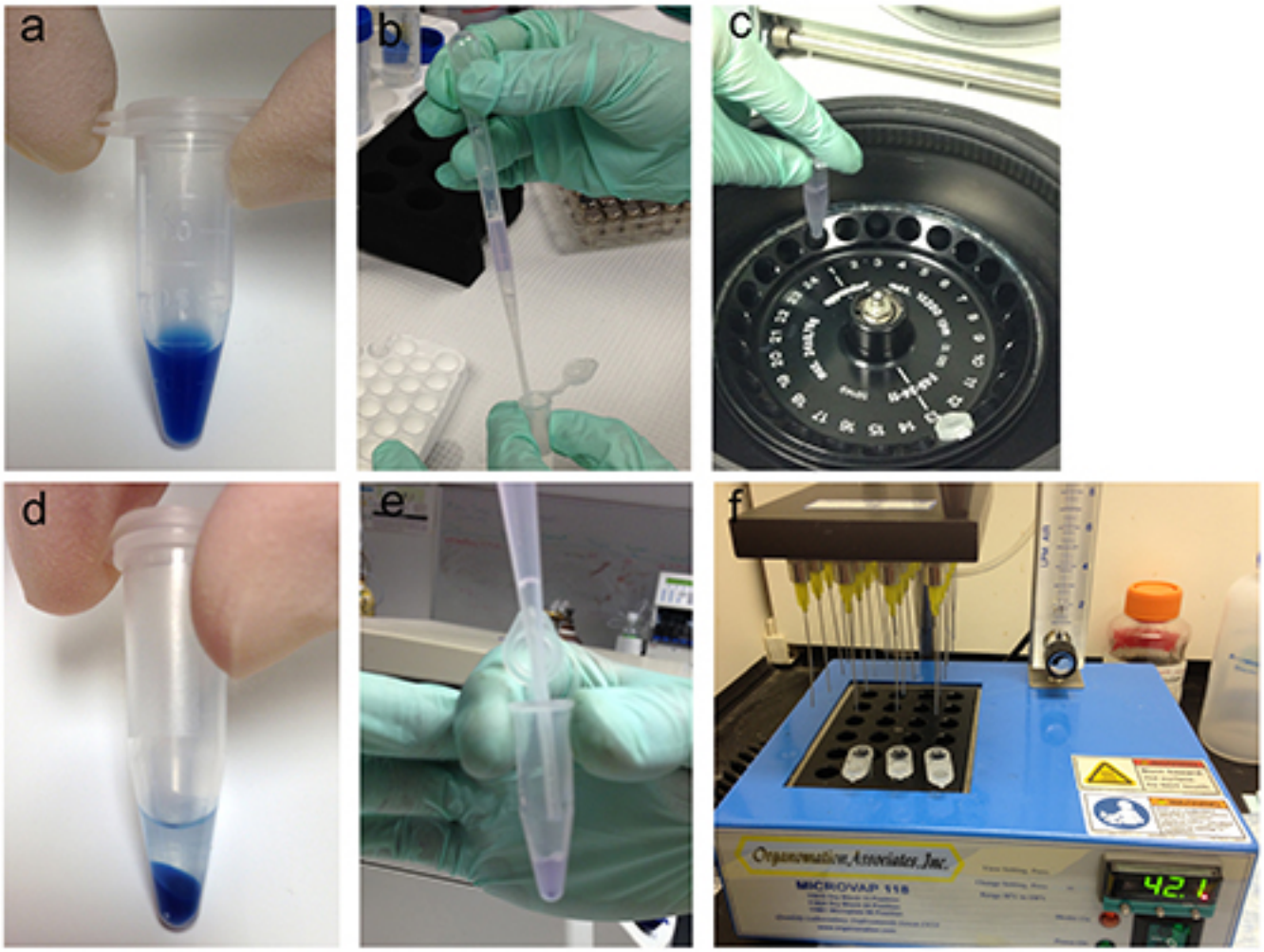

Figure 2. Nanoparticle harvesting procedure from plasma/serum for downstream mass spectrometry analysis. (A) Nanoparticles remain suspended in solution (Poly(NIPAm/dye) shown). **(B)** Nanoparticles are added to the diluted plasma sample. **(C)** The nanoparticle-plasma suspension is spun in a centrifuge to separate the nanoparticles from the supernatant. **(D)** Post centrifugation, the nanoparticles form a distinct pellet in the bottom of the tube. **(E)** After washing the nanoparticles, the eluate containing the harvested proteins is removed and saved for downstream analysis. **(F)** The eluate is dried under nitrogen flow in an evaporator at 42.1 °C, air flow 8, for 1 - 2 hr prior to mass spectrometry. Please click here to view a larger version of this figure.

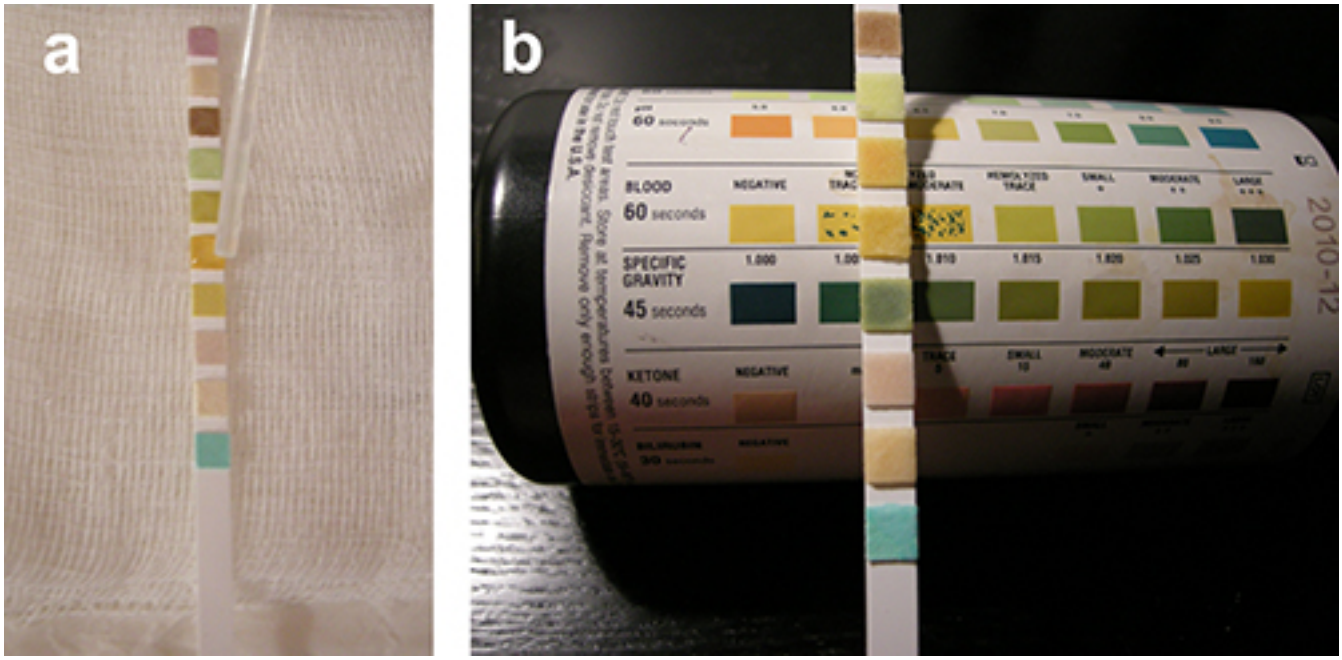

Figure 3. Urinalysis using multi-analyte reagent strips for specimen quality control. (A) Each pad on the reagent strip is impregnated with chemicals, enzymes, and/or indicator dyes which react with a different urine analyte (*e.g.*, glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrite, and/or leukocyte esterase). Urine is clarified by centrifugation. A drop of urine is added to each pad on the reagent strip. **(B)** The color of each pad is compared visually to the color blocks on the container, at the specified time. Please click here to view a larger version of this figure.

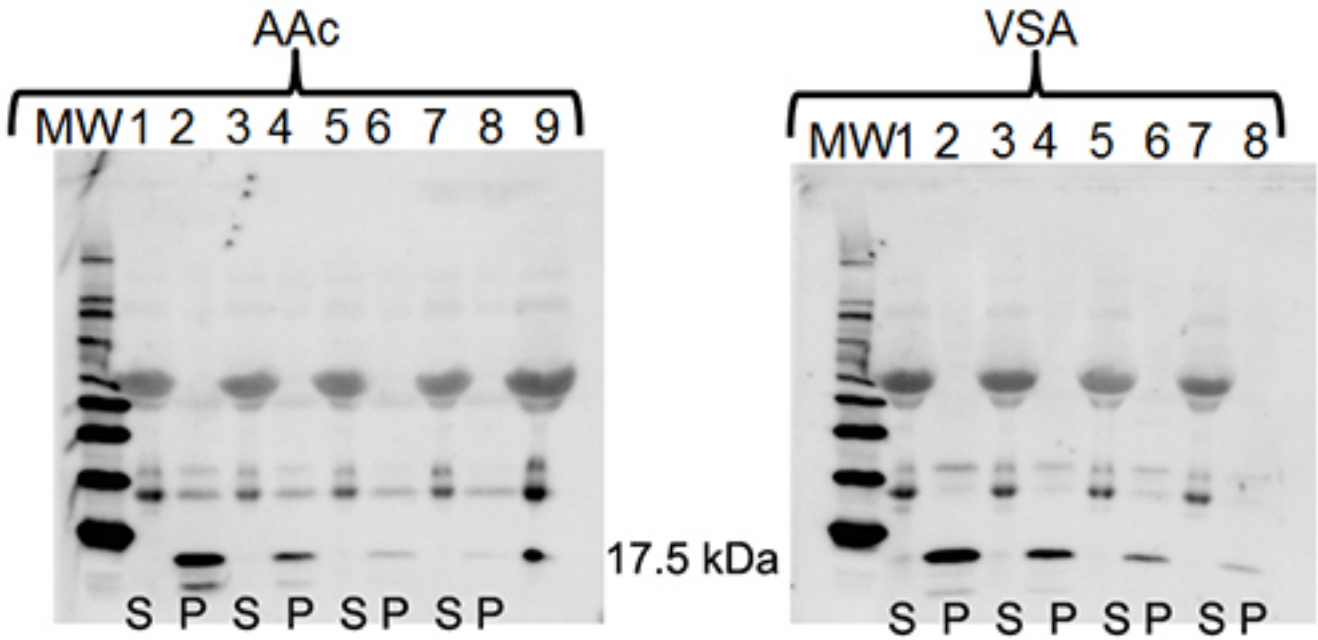

Figure 4. Harvesting IL-17 from plasma using core (AAc) or core-shell (VSA) nanoparticles. 1-D gel electrophoresis and western blotting with anti-IL-17 demonstrates the ability of Acrylic Acid (AAc) and VSA core shell nanoparticles to harvest various concentrations of IL-17 from plasma. (S = supernatant after nanoparticle harvesting, P = nanoparticle eluate. AAc particles: Lanes 1 & 2 = 1 ng/µl IL-17, Lanes 3 & 4 = 0.50 ng/µl, Lanes 5 & 6 = 0.25ng/µl, Lanes 7 & 8 = 0.125 ng/µl, Lane 9 = IL-17; VSA particles: Lanes 1 & 2 = 1 ng/µl IL-17, Lanes 3 & 4 = 0.50 ng/µl, Lanes 5 & 6 = 0.25 ng/µl, Lanes 7 & 8 = 0.125 ng/µl) Please click here to view a larger version of this figure.

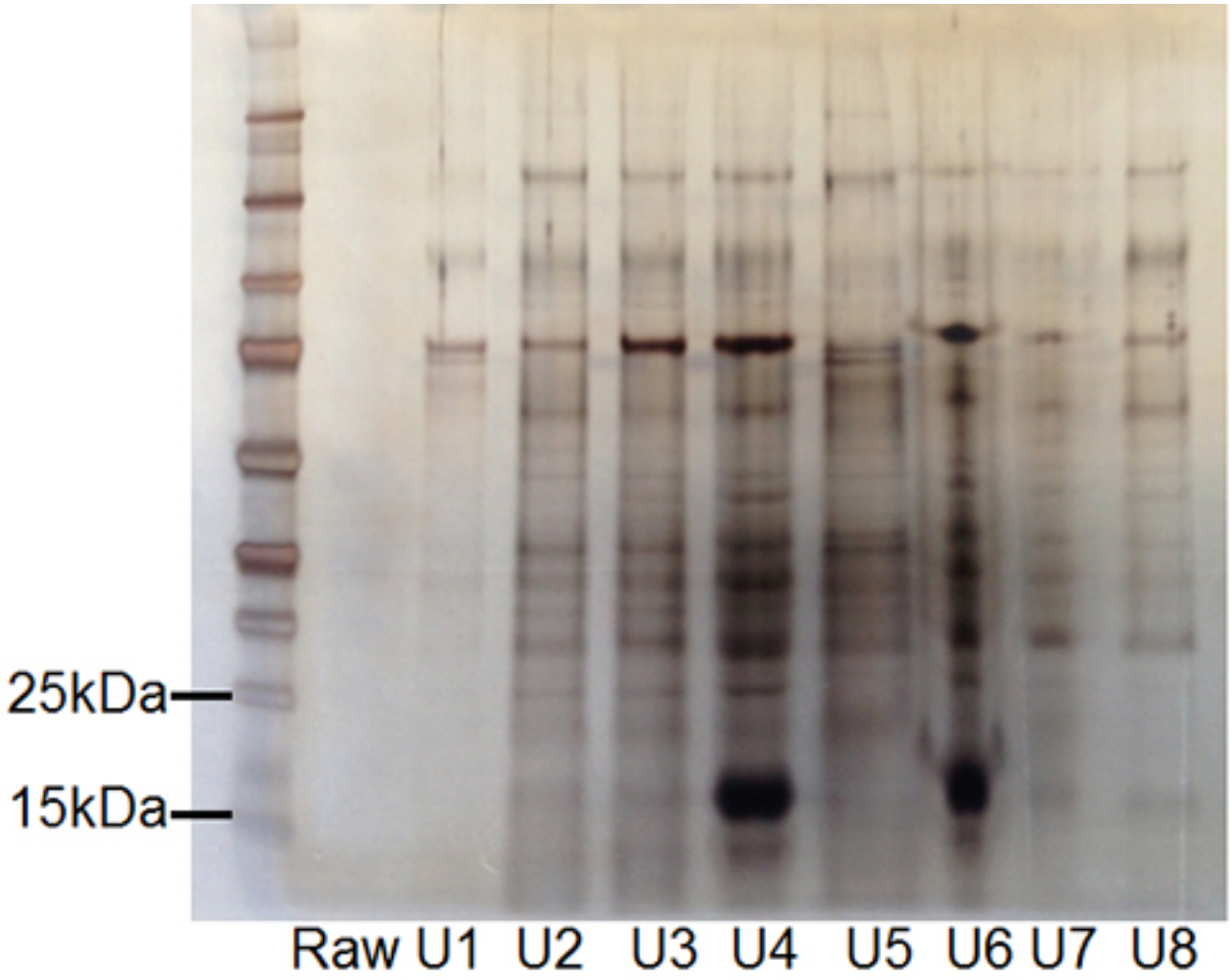

Figure 5. Recovery of *Mycobacterium tuberculosis* antigen and cytokine IL-2 from urine using hydrogel nanoparticles. Silver staining following 1-D gel electrophoresis of urine samples. Samples in lanes U4 and U6 represent eluates from urine samples containing recombinant ESAT-6 and IL-2, clearly showing prominent bands at 15 kDa. Mass spectrometry of the eluates identified ESAT-6 (UniProt accession POA567, 84.0 coverage, 9 peptides) and IL-2 (UniProt accession P60568, 38.56 coverage, 3 peptides). (RAW=urine without nanoparticle harvesting; U1, U2, U3, U5, U7, U8 = urine lacking recombinant ESAT-6 and IL-2 following nanoparticle harvesting). Please click here to view a larger version of this figure.

Protein name	GI protein number	Concentration in serum / plasma [ng/ml]	Reference
abl-interactor 1 isoform a	61743942	Unknown	
AMP-activated protein kinase gamma2 subunit isoform a	33186925	Unknown	
Cas-Br-M (murine) ecotropic retroviral transforming sequence	52426745	Unknown	
chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	4506831	30	34
chemokine (C-C motif) ligand 5	22538814	1.5	31
chondroadherin	153251229	Unknown	
chromosome 16 open reading frame 80	8392875	Unknown	
Consortin	213021160	Unknown	
defensin, alpha 4, corticostatin	4503303	Unknown	

EF-hand domain family, member D2	20149675	Unknown	
glycoprotein Ib (platelet), beta polypeptide	4504073	Unknown	
guanine nucleotide binding protein (G protein), q polypeptide	40254462	Unknown	
Heparanase	148746204	Unknown	
insulin-like growth factor 2 (somatomedin A)	189083846	100	30
lacritin	15187164	Unknown	
leukocyte cell-derived chemotaxin 2	59806345	200,000	33
lipocalin 2 (oncogene 24p3)	38455402	0.05	28
Monoglyceride lipase, isoform CRA_b	6005786	Unknown	
N-ethylmaleimide-sensitive factor attachment protein, alpha	47933379	Unknown	
one cut homeobox 2	119220564	Unknown	
outer dense fiber of sperm tails 4	171184433	Unknown	
palate, lung and nasal epithelium associated	18765705	Unknown	
peptidoglycan recognition protein 2	156616294	Unknown	
platelet-derived growth factor alpha polypeptide	77695917	4	29
Protein CASC3	15721939	Unknown	
RAB27B, member RAS oncogene family	5729997	Unknown	
Ras association (RalGDS/AF-6) domain family 6 isoform a	29789443	Unknown	
ras-related GTP-binding protein RAB10	33695095	Unknown	
ring finger protein 166	30520320	Unknown	
serum deprivation response (phosphatidylserine binding protein)	4759082	Unknown	
solute carrier family 33 (acetyl-CoA transporter), member 1	4757708	Unknown	
ST6 beta-galactosamide alpha-2,6-sialyltranferase 1 isoform a	27765091	15	32
thymosin-like 3	34013530	Unknown	
transducin-like enhancer of split 3 (E(sp135) homolog, Drosophila)	157384982	Unknown	
transglutaminase 1	4507475	Unknown	
WD repeat domain 1	9257257	Unknown	
WD repeat domain 91	222080092	Unknown	
xin actin-binding repeat containing 2	119372317	Unknown	

Table 1. Example low abundance proteins harvested by nanoparticles from serum/plasma. (PeptideAtlas peptidome mass spectrometry)[28-34]. Adapted with permissionfrom reference [13](Tamburro, D. *et al.*Multi-functional core-shell nanoparticles: Discovery of previously invisible biomarkers. *J Am Chem Soc*, **133**, 19178-19188, (2011).) Copyright 2011 American Chemical Society.

Discussion

Clinical Relevance

A serum or plasma sample is thought to contain low-abundance circulating proteins and peptides which can provide a rich source of information regarding the state of the organism as a whole. Despite the promise of serum proteomics, there are three fundamental and serious physiologic barriers thwarting biomarker discovery and translation to clinical benefit[10, 11, 16, 25].

1. Important diagnostic biomarkers may exist in extremely low-abundance (concentration) in blood. Early stage diseased tissue, such as pre-metastatic cancer lesions, may constitute less than a few mm^3. Biomarkers shed into the circulation from such a small tissue volume will become highly diluted in the entire blood volume. Relevant analytes may exist below the detection limits of mass spectrometry and conventional immunoassays.

2. Low abundance biomarkers/proteins are masked by the presence of high abundant proteins such as albumin and immunoglobulin, which represent up to 90% of the plasma proteome[14].

3. Proteins and peptides are susceptible to degradation by endogenous and exogenous proteinases following venipuncture and sample transport/storage. Protein degradation can lead to false positive or false negative results[35].

Hydrogel nanoparticles were developed as porous, buoyant, polymers containing anthraquinonetriazine dyes and/or vinylsulfonic acid shells for protein and peptide harvesting and preservation in body fluids[11, 13]. Our group synthesized and tested hydrogel nanoparticles functionalized with a plethora of organic dyes (*i.e.*, sulfonated and non-sulfonatedanthraquinonetriazine dyes) and showed the preferential affinities for several protein analytes[11, 13]. We also established protocols for biomarker discovery that use hydrogel nanoparticles with lymph, saliva, cerebrospinal fluid, sweat, urine, plasma, blood, or serum specimens[11, 13, 23, 24, 26].

Optimizing the nanoparticle harvesting parameters prior to harvesting proteins from precious clinical/research specimens is necessary for ideal results. The amount of protein in the samples should be quantified to determine the optimum ratio of nanoparticles to sample volume. For analytes of unknown concentration, a dose-response curve using recombinant proteins provides information regarding the limits of detection. If there is adequate sample, various types of nanoparticles can be used to determine the ideal nanoparticle for the analyte and specimen.

Urinalysis prior to nanoparticle harvesting provides a urine sample quality control check. The affinity of the nanoparticle dye bait depends on the isoelectric point of the protein of interest and the pH of the surrounding medium. Urine pH between 5.5 and 7.0 is optimal for protein harvesting with the poly(NIPAm/dye) nanoparticles. The presence of hemolyzed or intact red blood cells (1+ blood on the reagent strip) does not interfere with nanoparticle harvesting.

In this protocol we focused on the application of the hydrogel nanoparticles to harvest proteins from plasma and urine. Future applications could comprise other body fluids such as vitreous of the eye, or synovial fluid. Potential *in vivo* applications can be envisioned for the future in which nanoparticles are injected into diseased tissue to collect biomolecules. Future work will also be focused on the development of new devices for the capture and preservation of biomarkers, such as a nanoparticle-based skin patch for the analysis of the skin transudate proteome.

Disclosures

Benjamin Espina is an employee of Ceres Nanosciences, Inc. that produces reagents used in this Article. Lance Liotta, Alessandra Luchini and Virginia Espina hold patents (US patent 7,935,518 and/or 8,497,137) on the nanoparticle technology used in this Article. As university employees they are entitled to receive royalty from these patents per university policy. Lance Liotta and Alessandra Luchini are shareholders in Ceres Nanosciences and serve on the Scientific Advisory Board.

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