Magnetization of Hydrogel Particles: a Novel Urine Tuberculosis Antigen Test.

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ABSTRACT

This study aims at developing a nanotechnology-based screening test that would reliably detect early stage tuberculosis (TB) infection and discriminate active from latent infections. The use of urine in TB testing has been hampered by 1) extremely low concentration analytes and 2) protein lability. A novel urine collection system based on bait functionalized hydrogel nanoporous particles is developed that in few instants concentrates the analytes present in a large volume of urine (e.g. 30 mL) in a small volume of bait functionalized particles (e.g. 50 ul) and protects the captured analytes from degradation. Hydrogel particles can be shipped to the analysis laboratory at room temperature without the need of costly freezing preservation. A novel separation method based on magnetic nanoparticle tags permits the harvesting of hydrogel particles in few seconds without using high speed centrifugation. This technology can enable the production of an urinary TB antigen test applicable in developing country settings where the access to conventional laboratory equipment might be limited.

Keywords: hydrogel particles, biomarkers, infectious diseases, urine, diagnostics

1 INTRODUCTION

A critical unmet goal of the biomedical research sector is the identification of low abundance biomarkers that correlate with therapeutic outcome or toxicity, or that are predictive of early stage disease [1]. Despite a growing interest in the value of biomarkers, the research investment in biomarker discovery and clinical validation has not met the expectations to date [1, 2]. This poor return is due in large part to the low abundance of early disease biomarkers that exist at a concentration below the detection limit of biomarker discovery platforms.

Protein biomarker discovery and quantitation by mass spectrometry (MS) and multiple reaction monitoring (MRM) are powerful approaches [1, 2] but are severely limited in their practical application because of technical and physiological constraints [3]:

1) The vast majority of diagnostic analytes measured in the clinical laboratory by immunoassays has a concentration range (50 pg/mL and 10 ng/mL [4]) much lower than the analyte detection sensitivity for MS or MRM applied directly to a complex body fluid (>50 ng/mL) [5]. Thus, the most important protein biomarkers, particularly those derived from early stage disease [6], are invisible to conventional MS or MRM [7].

2) Proteins and peptides are masked by billion fold excess quantities of resident proteins such as immunoglobulin and albumin. The MS input sample is strictly limited in the maximum total protein (< 50 µg) content, a value lower than the serum or plasma protein content in the microliter volume of the MS input. If the sample is simply concentrated, this will overwhelm the total protein capacity of the MS.

3) candidate biomarkers are very perishable following clinical sample collection. Biomarker instability is a serious problem that has hindered the utility of biospecimens and prevented validation [2].

To overcome these barriers, we created novel nanoporous, buoyant, core-shell hydrogel particles containing novel reactive high affinity chemical baits. In one step following addition to a complex biologic fluid, our particles affinity harvest, preserve, and concentrate desired analytes, while simultaneously excluding unwanted high abundance proteins (Figure 1).

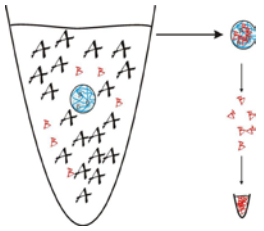


Figure 1. Biomarker harvesting: particles carrying affinity bait are mixed in a tube of blood (black *A*s represent albumin and other carrier proteins, red *B*s represent biomarkers). When particles are isolated from blood they contain biomarkers of interest that are eluted out of particles and are collected in a smaller volume, yielding high concentration and purification.

This new particle technology effectively protected highly labile proteins such as interleukins and growth factors from enzymatic degradation in blood, urine and sweat, and massively increased the effective detection sensitivity, while improving the precision, of multiple reaction monitoring (MRM) analysis [8, 9]. Used in whole blood as a one step, in-solution preprocessing step, the nanoparticles greatly enriched low molecular weight proteins and peptides while excluding unwanted albumin

and immunoglobulins; this achieved a 10,000 fold improvement in the lower limit of mass spectrometry (MS), permitting the discovery of hundreds candidate biomarkers that were previously undetectable in the nanogram/mL and picogram/mL range [8, 9].

Urine is a body fluid that can be sampled non-invasively and has the potential to contain early markers for diseases targeting any tissue [10, 11]. Despite this promise, urine is a difficult biologic fluid for biomarker discovery for the following reasons: a) Collection and storage volumes are large (~30 to 50 mL). These large volumes place considerable strain on resources for collection, shipment and storage, particularly for collection in the field, or in community based clinics. b) Urine is highly perishable, is rapidly contaminated with bacteria, and urinary analytes are rapidly degraded during room temperature storage [12], c) The protein concentration and specific gravity of urine varies widely depending on the patient's hydration state and kidney function, and, d) Centrifugation of large volume samples of urine are cumbersome and appropriate equipment is usually not available in community clinics.

We propose to use the bait functionalized particle technology for urine biomarker discovery and measurement. We propose a new format for urinary biomarker harvesting using magnetized particles. Particles introduced into the urine in the collection vessel immediately sequester and preserve analytes from the entire volume of urine. In a user-friendly format a magnet can be held against the collection vessel to collect the nanoparticles in a small volume. The urinary biomarkers, captured and preserved in a small volume of nanoparticles (50uL) can be easily stored, or mailed, for later analysis. We magnetized the particles by the introduction of a novel magnetic labeling reagent that is sequestered by the hydrogel particles after the urine analytes are captured. Simple addition of this labeling nanoparticle reagent magnetizes the harvesting particles so that they can be readily immobilized by a hand held magnet.

2 MATERIALS AND METHODS

2.1 Nanoporous Particle Fabrication

Core Shell Nanoparticle Fabrication: Carboxylic group containing hydrogel nanoparticles, poly(N-isopropylacrylamide (NIPAm)-co-acrylic acid (AAc)) was created by precipitation polymerization and covalently functionalized with one of organic reactive dyes by amidation reaction between the carboxylic group in the nanoparticles and the amine group in the dye. An outer shell containing vinylsulfonic acid (VSA) co-polymer was created on the dye functionalized particles by a second polymerization reaction as described in our previous publications [8, 9].

2.2 Particle Incubation with Model Solution, Serum, and Urine and Analytical Measurement

Aliquots model solution, serum and urine were mixed with bait functionalized particles and incubated for 15 minutes at room temperature. Particles were separated by centrifugation (16.1 rcf, 25 °C, 10 minutes) and washed acqueous buffers. Proteins were eluted with 600 uL of elution buffer (70% acetonitrile, 10% ammonium hydroxide). Eluates were dried under nitrogen flow and subjected to analytical measurements. Particle eluates were analyzed with mass spectrometry, SDS PAGE, western blot and ELISA as described in our previous publications [8, 9].

3 RESULTS

3.1 Bait Functionalized Particle Attributes.

Quick Uptake: Bait functionalized particles were incubated with model solutions containing lysozyme (0.075 ug/ul), bovine serum albumin (BSA, 0.075 ug/ul), and platelet derived growth factor (PDGF, 0.075 ug/ul) dissolved in 50 mM TrisHCl, pH 7. Time course studies were performed in order to determine the timing of the association/dissociation kinetics. As shown in Figure 2A, as soon as 1 minute after incubation, all the low molecular weight proteins (lysozyme and PDGF) were completely sequestered from the supernatant while high molecular weight BSA is not captured by the particles. After one hour all the LMW proteins are still bound to the particles, demonstrating low dissociation rates (Figure 2A).

Sharp Molecular Weight Cut Off: Bait functionalized particles were incubated with model solutions containing BSA (0.075 ug/ul, 66 kDa), myoglobin (0.075 ug/ul, 17 kDa), insulin (0.075 ug/ul, 6 kDa), PDGF (0.075 ug/ul, 17 kDa), aprotinin (0.075 ug/ul, 6 kDa), trypsin inhibitor (0.075 ug/ul, 21 kDa), carbonic anhydrase (0.075 ug/ul, 30 kDa), ovalbumin (0.075 ug/ul, 45 kDa) dissolved in 50 mM Tris HCl, pH 7. SDS PAGE and flow cytometry analysis demonstrated that proteins with a molecular weight lower than 21 kDa were sequestered inside the particles, whereas all the proteins of higher molecular weight were excluded (Figure 2B).

Protection from degradation: Model solutions were prepared dissolving lysozyme (0.075 ug/ul) and PDGF (0.075 ug/ul) in 50 mM Tris HCl, pH 7. Excess trypsin (1:50 w/w protein:protease ratio) was added to the protein solutions and incubated for different times (10, 20, 40, 60 minutes and overnight) at 37˚C. Degradation was assessed by SDS PAGE and western blot analysis (Figure 2C) and resulted in a fainter band for the intact protein and appearance of bands migrating faster than the intact protein. In order to assess the preservation attribute, bait functionalized particles were mixed to protein/protease solutions and let incubate for the same timing described above (10, 20, 40, 60 minutes and overnight). SDS PAGE and western blot analysis demonstrated that bait functionalized particles fully protect from degradation captured proteins, as demonstrated by the lack of products of degradation (Figure 2C).

Capture of native analytes and antigenicity preservation: Bait functionalized particles were incubated with 500 ul of human serum diluted 1:2 with 50 mM Tris HCl, pH 7 for 30 minutes. Original serum (50 ul) and particle eluates (50 ul) were analyzed with ELISA directed against PDGF and PDGF western blotting. Results shown in Figure 2D demonstrated that bait functionalized particles captured all the solution phase, native PDGF and the recovery yield was higher than 95% in both ELISA and western blot analysis.

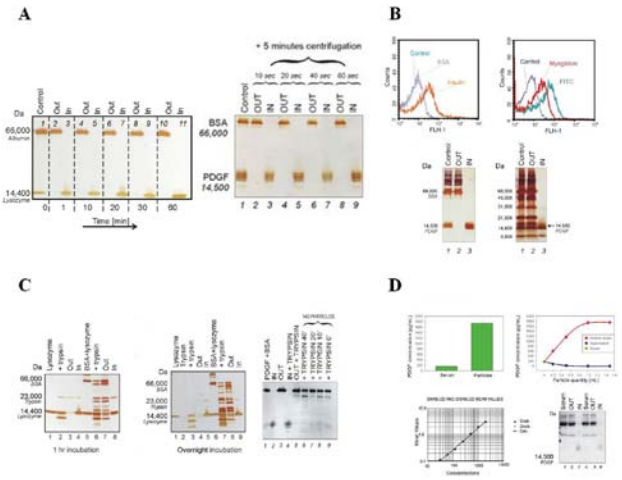


Figure 2: Hydrogel particles perform A) quick and complete uptake of solution phase target analytes, B) sharp molecular size sieving, C) protection of degradation of captured molecules even in presence of excess proteases, and D) capture and preservation of antigenicity of native proteins.

3.2 Mathematical Modeling of Protein-Particles Interaction Kinetics.

Theoretical analysis. The feasibility of using custom designed bait particles to harvest and sequester low molecular weight or low abundance biomarkers, even though the biomarker is complexed with high abundance proteins (e.g. albumin), can be demonstrated by the following simple mathematical model. At *t=0*, a harvesting capture molecule is introduced to the system, at which time an equilibrium is assumed to exist between the free (uncomplexed) biomarker, *b*, the 'natural' capture carrier protein, C_N, and the biomarker-carrier protein complex, bC_N. In the absence of a harvesting carrier molecule, the reaction scheme is as depicted below,

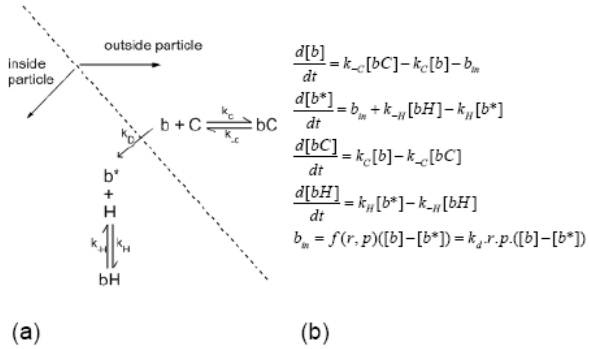
$$b + C_N \underset{k_{-N}}{\overset{k_N}{\rightleftharpoons}} bC_N$$

where k_N and k_{-N} are the forward and reverse rate constants for the reaction, respectively. Here it is assumed that the carrier protein exists in such vast excess over the biomarker that the free carrier protein concentration, $[C_N]$, may be considered a constant ($[C_N] >> [bC_N] \;\forall\; [bC_N] \Rightarrow [C_N] \approx [C_N]$), and may be absorbed into the forward rate constant. Thus, the equilibrium ratio will be given by

$$\frac{[bC_N]}{[b]} = \frac{k_N}{k_{-N}} = K_N,$$

$$b + C_N \underset{k_{-N}}{\overset{k_N}{\rightleftharpoons}} bC_N$$

where K_N is the affinity constant for the biomarker and its natural carrier protein. Note that $k_N >> k_{-N}$ or $K_N >> 1$ since the relatively large free carrier protein concentration typically amplifies the forward reaction rate by several orders of magnitude [13]. When the harvesting capture particle is introduced, on the other hand, the reaction scheme is as depicted in Figure 3, where k_H and k_{-H} are the forward and reverse rate constants, respectively, for the reaction between the biomarker and the harvesting capture particle, C_H, producing the complex bC_H.. Note that the rate constants k_H and k_{-H} account for the rate of biomarker transit through the particle pores as well as the affinity of the biomarker for the encapsulated bait molecule.



(a) (b)

Here again, the harvesting particles are present in such vast excess over the low abundance biomarker that their (very large) concentration may be absorbed into the forward rate constant, k_H, so that $k_H >> k_{-H}$ The introduced harvesting particles have a much greater *innate* affinity for the small biomarkers, in addition to an existing at a higher concentration compared to the natural carrier proteins, so that $k_H >> k_N$. For this reason, an association between the biomarker and the harvesting particle is markedly preferred over the complex with the natural carrier protein. Since the harvesting particle concentration itself contributes to the forward rate constant, k_H, this bias towards the harvesting particle will be enhanced further as the concentration of harvesting particles in the mixture is increased. The temporal variations of the free and complexed forms of the biomarker are now described by the suite of Equations (2)

through (4) below, subject to the specified initial conditions and parameter set.

The mechanism of analyte removal from carrier proteins during the process of bait capture can be explained by these model solutions as follows: When the harvesting particles are first introduced to the mixture, the bound and free-phase biomarkers are initially in the ratio determined by Equation (1), i.e., $[bC_N]=K_N[b]$. The harvesting particles immediately begin to sequester the free-phase biomarker, attempting to establish the corresponding ratio $[bC_H]=K_H[b]$ between the complexed and uncomplexed biomarker forms.

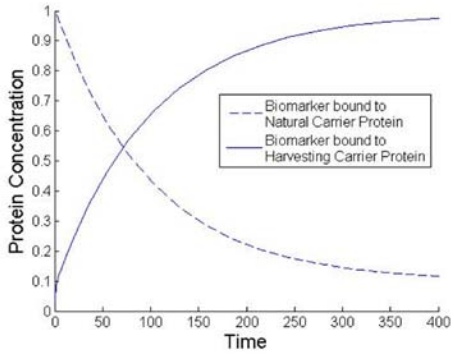


Figure 3. Transfer of a low molecular weight biomarker from a natural carrier protein to a harvesting particle with ten times greater affinity for the biomarker.

This removal of free-phase biomarker from the mixture perturbs the equilibrium ratio for the natural carrier protein, so that $[bC_N]<K_N[b]$, which in turn generates a driving force for the transfer of biomarker from the natural carrier protein to the free-phase form. This transfer sponsors the continued binding of free-phase biomarker to the harvesting particles. Biomarker transfer from the natural carrier protein to the harvesting particle via the free-phase form will continue in this way until an overall equilibrium is reached, with $[bC_H] = K_H[b]$ and $[bC_N]=K_N[b]$. The proportion of biomarker in complexed association with the harvesting particle will then be $K_H/(1+K_N+K_H) \approx K_H/(K_N+K_H)$. Thus, the greater the biomarker affinity for the harvesting particle, K_H, in comparison with its affinity for the natural carrier protein, K_N, the closer this ratio will be to unity (ie. *all* biomarker in complexed association with the harvesting particle.)

3.3 Bait Functionalized Particles Enahnced Biomarker Discovery by Mass Spectrometry.

A set of 40 serum samples from consented healthy volunteers was subjected to particle processing and mass spectrometry (MS) analysis. Proteins identified were compared with the list of proteins identified by MS analysis of crude serum (Figure 4). MS analysis without the use of bait functionalized particles yielded 87 proteins, all with a concentration in serum > 50 ng/mL. MS analysis of particle-pre processed serum yielded 258 total proteins, 88 of which have a known concentration in serum <50 ng/mL (as measured by immunoassays) or were previously unknown to exist in serum (Figure 4).

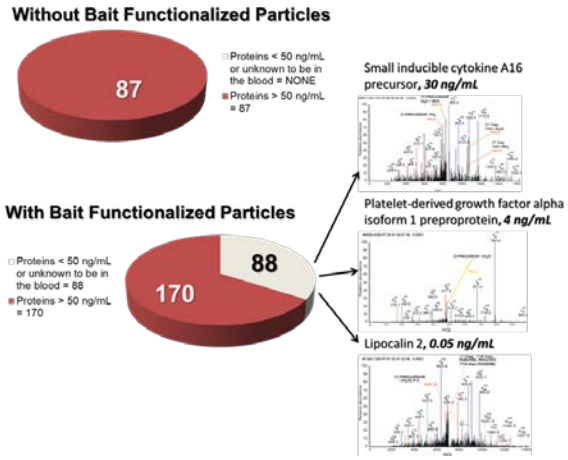


Figure 4: Bait functionalized particles massively enhanced the effective sensitivity of mass spectrometry analysis of serum. Upper panel: all proteins identified in crude serum have concentration higher than 50 ng/mL. Lower panel: 88 out of 258 proteins identified with MS analysis after particle pre-processing have concentration lower than 50 ng/mL or were previously unknown to exist in serum.

4 CONCLUSIONS

Bait functionalized, harvesting nano-porous particles are an enabling technology that can maximize the utility of biofluids for molecular analysis. We propose to apply this novel technology to the problem of early diagnosis of infectious diseases such as tuberculosis.

REFERENCES

1. Aebersold, R. and M. Mann, Nature, 2003. **422**(6928): p. 198-207.
2. Poste, G., Nature, 2011. **469**(7329): p. 156-7.
3. Gerszten, R.E., et al., Am J Physiol Lung Cell Mol Physiol, 2008. **295**(1): p. L16-22.
4. Anderson, N.L. and N.G. Anderson, Mol Cell Proteomics, 2002. **1**(11): p. 845-67.
5. Kuhn, E., et al., Clin Chem, 2009. **55**(6): p. 1108-17.
6. Lutz, A.M., et al., PLoS Med, 2008. **5**(8): p. e170.
7. Pan, S., et al., J Proteome Res, 2009. **8**(2): p. 787-97.
8. Luchini, A., et al., Nano Lett, 2008. **8**(1): p. 350-61.
9. Tamburro, D., et al., J Am Chem Soc, 2011. **133**: p. 19178-19188.
10. Laxman, B., et al., Cancer Research, 2008. **68**(3): p. 645-649.
11. Chambers, A.F. and B.C. Vanderhyden, Clinical Cancer Research, 2006. **12**(2): p. 323-327.
12. CA, B., A. ER, and B. DE, 2012, St. Louis Miss: Saunders Elsevier.
13. Lowenthal, M.S., et al., Clin Chem, 2005. **51**(10): p. 1933-45.