BAIT LOADED HYDROGEL PARTICLES PERFORM ONE STEP, IN SOLUTION SEQUESTRATION, CONCENTRATION AND PROTECTION FROM DEGRADATION OF LOW MOLECULAR WEIGHT, LOW ABUNDANCE DISEASE BIOMARKERS IN BLOOD AND URINE

Alessandra Luchini*, Caterina Longo, Claudia Fredolini, Francesco Meani, Davide Tamburro, Virginia Espina, Palma J Botterell, Alexis Patanarut, Barney Bishop, Weidong Zhou, Mark M Ross, Emanuel F Petricoin III, Lane A Liotta
George Mason University, VA.
*aluchini@gmu.edu

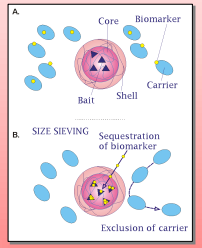
ABSTRACT

Disease-associated blood biomarkers exist in exceedingly low concentrations in the blood and urine and may be subjected to degradation during transportation and storage as a result of endogenous or exogenous proteinase activity. We have produced a N-isopropylacrylamide core shell and sieving nanoparticle with an affinity bait molecule that performs three independent functions within minutes, in one step, in solution, in serum and urine: a) molecular size sieving b) affinity capture of all solution phase target molecules, and c) complete protection of harvested proteins from enzymatic degradation. N-isopropylacrylamide/acrylic acid (NIPAm/AAc) particles isolated low molecular weight, low abundance polypeptides and completely excluded albumin in a variety of known molecular mixtures. The low abundance biomarker platelet derived growth factor (PDGF, 14,000 Da) was completely sequestered from serum and concentrated within the nanoparticles for a concentration factor of many hundred fold depending on the starting volume. Flow cytometry, western blot, mass spectrometry, and ELISA studies demonstrated that particles sequestered and concentrated a variety of proteins and metabolites which had a size smaller than the molecular sieve cut-off and were recognized by the bait. NIPAm/AAc particle completely blocked protease degradation of captured proteins even at 37°C overnight, using two different proteases, trypsin and chymotrypsin, at high concentration (1:20).

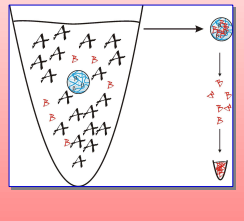
NIPAm particles loaded with a different bait molecule, Cibacron blue F3G-A (CB), successfully captured, preserved and concentrated one of the most difficult low abundance, but medically relevant, hormones in the urine: human growth hormone (hGH). Both exogenous hGH, spiked in synthetic and human urine, and endogenous hGH from healthy donor urine, were sequestered by the NIPAm/CB particles, and measured with a clinical immunometric assay (IMMULITE-Siemens).

This nanotechnology appears to protect putative biomarkers from degradation with the desired precision and sensitivity to support large scale biomarker discovery and measurement studies.

INTRODUCTION



Schematic representation of particle structure and function. (a) Particles are constructed with a bait containing core, surrounded by a sieving shell. (b) When introduced into a complex solution, such as serum, core-shell particles perform affinity capture of low-molecular-weight proteins from the carrier protein albumin and function as a molecular weight sieve with total exclusion of high molecular weight proteins

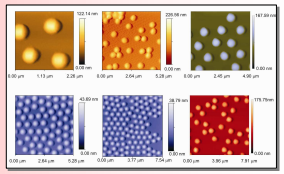


NIPAm-based particle in a tube of blood harvest biomarkers, which are then eluted in a smaller volume. In this way, biomarkers are concentrated and partially purified.

Bait	Target
Acrylic acid	Cationic proteins and polypeptides
Aldolase, 1-vinylimidazole, N,N'-(divinylurea)propyl methacrylate	Anionic proteins and polypeptides
Procion Red H8BN, Cibacron Blue F3G-A	Proteins and polypeptides
Cyclodextrin	Steroids, small molecules, cholesterol
P-vinylphenyl boronic acid, N-acrylo-m-aminophenylboronic acid (NAAPBA)	Polysaccharides, glycopeptides, RNA
TiO_2 nanoparticles incorporated in NIPAm beads	Phosphopeptides

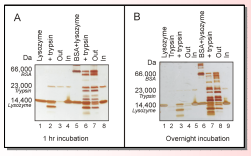
Summary of bait chemistries. Chemical formulas of the baits are shown in the left column, and the class of target molecules is reported on the right column. Baits include co-monomers (e.g., acrylic acid) or chemical moieties that are covalently bound inside the particle in a second reaction after polymerization (e.g. Cibacron Blue F3G-A).

HYDROGEL PARTICLES

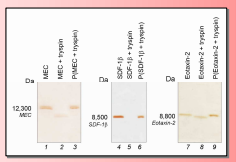


Atomic force microscopy images of NIPAm-AAc particles. Particles suspended in MilliQ water (pH 5.5, 1 µg/mL) were deposited on freshly cleaved mica under humid atmosphere at room temperature for 15 minutes and dried under nitrogen. Particles exhibit a uniform size distribution (diameter ~800 nm).

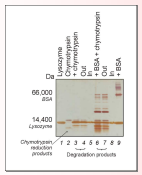
PROTECTION FROM DEGRADATION



SDS PAGE analysis demonstrating that tryptic degradation of lysozyme in its native form yielded clearly detectable products when conducted at 1:10 w/w protease:protein ratio and overnight incubation. **In the same conditions NIPAm/AAc particles harvested both the protein and the protease and fully protected lysozyme from degradation.** In the presence of BSA in solution, lysozyme and trypsin were captured by particles and lysozyme was protected from proteolysis, while BSA was left in solution and was vastly degraded by the remaining enzyme.

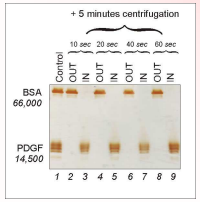


SDS PAGE analysis showing that core-shell particles protect chemokines from enzymatic degradation. Core-shell particles were incubated with the following chemokines, mucosae-associated epithelial chemokine (MEC/CCL28), stromal cell-derived factor-1 beta, (SDF-1b/CXCL12b), and eotaxin-2 (CCL24), in presence of trypsin. **Chemokines incubated with particles (Lane 3, 6, and 9) are protected from tryptic degradation** whereas chemokines not incubated with particles (Lane 2, 5, and 8) are susceptible to proteolytic digestion.



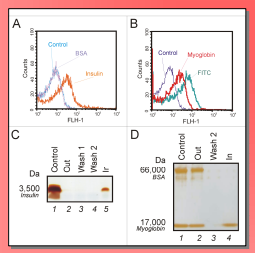
Bait particles protected lysozyme form chymotrypsin proteolysis. Lane 1) lysozyme, 2) chymotrypsin, 3) lysozyme + chymotrypsin, 4) lysozyme + chymotrypsin incubated with bait particles, supernatant (Out), 5) lysozyme + chymotrypsin incubated with bait particles, particles (In), 6) lysozyme + BSA + chymotrypsin, 7) lysozyme + BSA + chymotrypsin incubated with bait particles, supernatant, 8) lysozyme + BSA + chymotrypsin incubated with bait particles, particles (In), 9) lysozyme + BSA.

RAPID UPTAKE

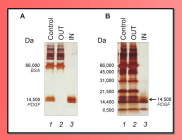


The kinetics of protein uptake was very rapid, in the order of minutes. Core shell particles were incubated with a solution containing PDGF and BSA for 10, 20, 40, and 60 seconds and centrifuged for 5 minutes. PDGF sequestration was nearly complete after 10 seconds. As expected, BSA was excluded by the particles.

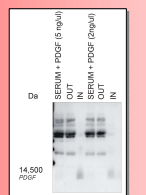
SEQUESTRATION AND CONCENTRATION



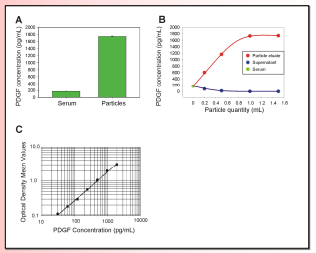
Bovine serum albumin (BSA, MW 66,000 Da) was completely excluded in all experiments, while smaller proteins such as insulin (MW 3,500 Da) and myoglobin (MW 17,000 Da) were harvested by particles as proven with flow cytometry and SDS PAGE analysis.



SDS-PAGE Analysis of PDGF incubated particles. (A) Particles harvest and concentrated PDGF and completely excluded BSA. (B) Particles harvest PDGF together with low molecular weight proteins and exclude proteins above ca 20,000 Da.



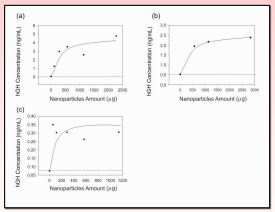
Western blot analysis revealed that recombinant PDGF spiked in human serum was barely detectable at two concentrations (5 and 2.5 ng/mL). NIPAm/AAc particles harvested and concentrated PDGF so that it became clearly detectable (approximately 300 times concentrated).



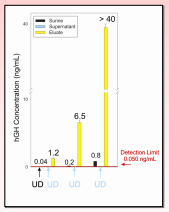
Core shell particles increased the concentration of native PDGF in serum as measured by ELISA assay. (A) ELISA readings of the starting serum solution in Calibrator diluent RD6-3 (R&D Systems, animal serum with preservatives) at a concentration of 170.91 +/- 4.66 pg/mL and PDGF eluted from core-shell particles (1743.43 +/- 11.06 pg/mL). (B) PDGF concentration in core-shell particle eluate plotted against the quantity of particles utilized for the incubation, duplicate experiments. (C) ELISA standard curve of PDGF concentration versus absorbance. The standard curve was generated with two repeats for each PDGF calibrator concentration.

Native PDGF was found in healthy donor serum purified by core shell particles and measured by nano reverse phase liquid chromatography mass spectrometry (RPLC-MS/MS). Fragments of PDGF were detected among a list of very rare and low molecular weight proteins. Sequest result filter criteria: Xcorr vs charge 2.2, 3.5 for 2+, 3+ ions; Delta Cn > 0.1, Top #1 ranked; P (Pro) < 0.01.

Reference	Accession[a]	P_{pep}[b]	S_C[c]	Score[d]	MW[e]	#Peptide
platelet-derived growth factor beta isoform 1, preproprotein	4505681	1.75E-05	0.91	10.15	27266.1	1
platelet-derived growth factor beta isoform 2, preproprotein	15451786	1.75E-05	0.91	10.15	25486.2	1

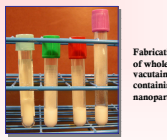


Particle dose response for capture of hGH: (a) Recombinant hGH was spiked in Surine and increasing amounts of particles were incubated with the analyte containing solution. The plateau is reached at about 600 µg. (b) Recombinant hGH was spiked in human urine and increasing amount of particles were added to the solution. The plateau was reached at about 600 µg. (c) Cadaveric hGH was spiked in human urine and recovered with particles. The plateau was reached with low amount of particles (600 µg).



Effective amplification of urine hGH immunoassay sensitivity. Dose response study on recombinant hGH spiked in 10 mL of Surine. Supernatants are depleted and hGH concentration in particles eluate is linearly dependent on hGH concentration in Surine. UD means undetectable (below the detection limits of the Immulite assay).

APPLICATION



Fabrication and testing of whole blood vacutainer tubes containing bait core shell nanoparticles

ACKNOWLEDGEMENTS

This work is supported by the NIH grant number 1R21CA137706-01