HYDROGEL PARTICLES PERFORM ONE STEP, IN SOLUTION SEQUESTRATION AND PROTECTION FROM DEGRADATION OF LOW MOLECULAR WEIGHT, LOW ABUNDANCE CANCER BIOMARKERS IN BLOOD

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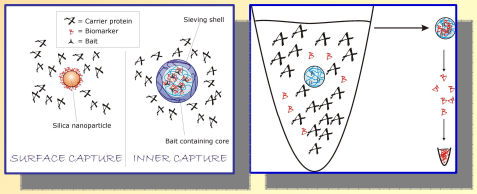
ABSTRACT

Cancer-associated blood biomarkers exist in exceedingly low concentrations within complex mixtures of high-abundance proteins such as albumin and immunoglobulins. Moreover, biomarkers in the blood may be subjected to degradation during transportation and storage. Such degradation is a significant source of bias for cancer biomarker measurement and discovery. We have created N-isopropylacrylamide porous, sieving, core shell "smart" nanoparticles containing an internal affinity bait to perform three independent functions within minutes, in one step, in solution (serum or plasma): a) molecular size sieving with complete separation from high abundance residence proteins such as albumin and immunoglobulin, b) affinity capture of all solution phase target molecules, and c) complete protection of harvested proteins from enzymatic degradation. The envisioned technology is a panel of dry lyophilized, sub-micron sized harvesting particles that carry specific bait for known biomarkers. Following introduction of the blood or body fluid, the respective particle populations will remove all of their target molecules, in one step, in solution, from the entire volume of the sample and concentrate the sequestered analytes inside the particles. Analytes will then be eluted from the particles to yield a much higher concentration and purification compared to the starting sample. Depending on the starting volume of the blood, this technology can concentrate a biomarker many hundred fold, and prevent degradation, within minutes.

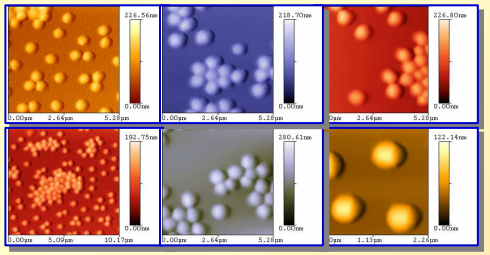
INTRODUCTION



Comparison of our approach to harvesting biomarkers in blood with other proposed methods involving nanoparticles[1,2,3]

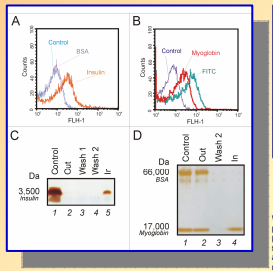
NIPAm-based particle in a tube of blood harvests and concentrates biomarkers, which are then eluted in a smaller volume

HYDROGEL PARTICLES

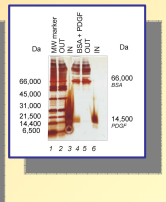


Atomic force microscopy pictures of NIPAm-based harvesting nanoparticles

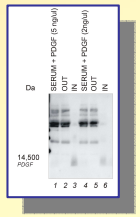
SEQUESTRATION AND CONCENTRATION



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

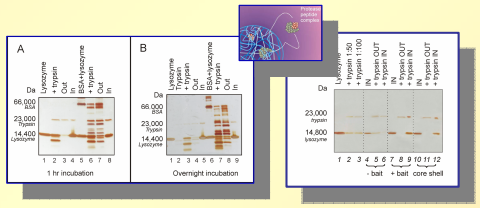


When NIPAm/AAc baited particles were incubated with a protein molecular weight marker solution, the particles captured and concentrated all protein molecules with MW less than ca. 21,500 Da, and did not bind any proteins with MW greater than 21,500 Da, with total exclusion of albumin



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).

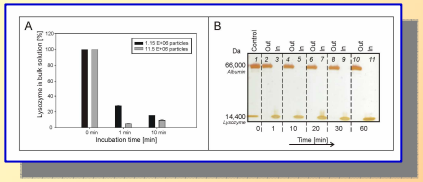
PROTECTION FROM DEGRADATION



SDS PAGE analysis demonstrated 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.

To verify the effectiveness of the bait to protect biomarkers from degradation, we used reduced and alkylated lysozyme and incubated it with trypsin and particles. **Particles** with bait harvested and concentrated denatured lysozyme and protected lysozyme from degradation.

RAPID UPTAKE



The kinetics of protein uptake was very rapid, in the order of minutes, for a lysozyme solution incubated with two different quantities of NIPAm/AAc particles. The amount of protein remaining in bulk solution after incubation with NIPAm/AAc particles was measured by Reverse Phase Protein Arrays (RPPA) The kinetics of protein uptake by NIPAm/AAc particles was further investigated by incubating particles with BSA and lysozyme using SDS-PAGE to monitor lysozyme uptake at time points of 1, 10, 20, 30, and 60 minutes. Lysozyme sequestration was nearly complete after 1 minute with no detectable change by 60 minutes, confirming that the process occurs very quickly as indicated in the flow cytometry time course study described above. As expected, BSA was excluded by the particles, and none of the BSA was taken-up by the NIPAm/AAc throughout the duration of the experiment (60 minutes)

BAIT STRATEGIES

Bait	Target
	Cationic proteins and polypeptides
	Anionic proteins and polypeptides
	Proteins and polypeptides
	Small molecules, cholesterol
	Polysaccharides, glycopeptides, RNA
TiO_2 nanoparticles incorporated in NIPAm beads	Phosphopeptides

NANOFLOW REVERSED-PHASE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

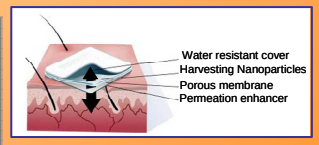
Recombinant PDGF (half life in serum = 3 minutes) was spiked in human serum at a concentration of 10 ng/ul and the serum was incubated for 1 hour with particles. Proteins were eluted from particles and analyzed with nanoRPLC-MS/MS. Fragments of PDGF were detected among a list of very rare and low molecular 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	P (pro)	SF	Score	MW	Accession	Peptide
hemopexin [Homo sapiens]	4.44E-16	1.84	20.23	51643.3	11321561	2
lysozyme precursor [Homo sapiens]	2.11E-14	4.54	50.25	16526.3	4557894	6
apolipoprotein H precursor [Homo sapiens]	1.38E-13	12.33	130.31	38286.7	4557327	17
hypothetical protein LOC649897 [Homo sapiens]	2.91E-13	2.92	30.26	22058.9	91206438	20
apolipoprotein C-III precursor [Homo sapiens]	3.36E-13	6.59	70.25	10845.5	4557323	15
platelet factor 4 variant 1 [Homo sapiens]	5.80E-13	3.80	40.24	11545.3	4505733	8
C-type lectin domain family 3, member B [Homo sapiens]	8.72E-13	6.16	70.28	22552.3	4507557	7
alpha-2-HS-glycoprotein [Homo sapiens]	1.36E-12	5.33	60.23	39299.7	4502005	16
pro-platelet basic protein precursor [Homo sapiens]	1.37E-12	8.29	90.28	13885.4	4505981	64
coagulation factor XII precursor [Homo sapiens]	2.02E-12	6.65	70.26	67774.1	4503629	7
properdin P factor, complement [Homo sapiens]	4.81E-12	2.86	30.24	51242.0	4505737	5
histidine-rich glycoprotein precursor [Homo sapiens]	5.01E-12	9.24	100.24	59540.9	4504489	12
clusterin isoform 1 [Homo sapiens]	1.25E-11	7.39	80.28	57795.7	42716297	10
pancreatic ribonuclease precursor [Homo sapiens]	3.28E-11	2.84	30.30	17832.7	38203682	3
vitronectin precursor [Homo sapiens]	1.11E-10	2.85	30.19	54271.2	88853069	3
gelsolin isoform b [Homo sapiens]	1.21E-10	6.54	70.23	80590.6	38044288	9
platelet-derived growth factor beta isoform 1, preproprotein [Homo sapiens]	3.79E-10	7.09	80.22	27266.1	4505681	25
platelet-derived growth factor beta isoform 2, preproprotein [Homo sapiens]	3.79E-10	7.09	80.22	25486.2	15451786	25
insulin-like growth factor 1 (somatomedin C) [Homo sapiens]	6.67E-10	0.96	10.21	17014.3	11034682	2
transthyretin [Homo sapiens]	7.26E-10	6.34	70.24	15877.1	4507725	7
alpha 1 type VIII collagen isoform 1 precursor [Homo sapiens]	9.46E-10	0.97	10.23	153745.9	13385630	1
fibrinogen, alpha polypeptide isoform alpha preproprotein [Homo sapiens]	8.68E-09	4.45	50.23	69713.8	11761629	5
selenoprotein P precursor [Homo sapiens]	1.04E-08	0.96	10.24	141646.7	62530391	1
lipocalin 2 (oncogene 24p3) [Homo sapiens]	1.32E-08	3.78	40.21	22573.7	38455402	4
serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 [Homo sapiens]	3.60E-08	1.91	20.24	46707.1	50363219	2
platelet factor 4 (chemokine (C-X-C motif) ligand 4) [Homo sapiens]	4.38E-08	4.76	50.24	10837.9	4505733	10
coagulation factor II precursor [Homo sapiens]	7.52E-08	0.97	10.21	69992.2	4503635	1
RAP1A, member of RAS oncogene family [Homo sapiens]	9.02E-08	0.97	10.20	20973.7	4506413	1
paraoxonase 1 [Homo sapiens]	2.54E-07	0.96	10.17	39706.3	19923106	1
orosomucoid 1 precursor [Homo sapiens]	3.28E-07	1.88	20.20	23496.8	9257232	2
peptidoglycan recognition protein 1 [Homo sapiens]	4.60E-07	0.91	10.17	21716.7	4827036	1
chemokine (C-C motif) ligand 14 isoform 1 precursor [Homo sapiens]	5.02E-07	2.79	30.22	10671.3	14589861	3
thrombospondin repeat containing 1 isoform 1 [Homo sapiens]	8.60E-07	0.71	10.12	116470.7	38016904	1

Reference	P (pro)	SF	Score	MW	Accession	Peptide
defensin, alpha 1 preproprotein [Homo sapiens]	1.22E-06	2.66	30.15	10194.2	4758346	5
small inducible cytokine A16 precursor [Homo sapiens]	1.31E-06	1.85	20.20	13591.4	4759074	2
defensin, beta 1 precursor [Homo sapiens]	1.42E-06	0.94	10.22	7414.6	4885381	1
dermokine isoform beta [Homo sapiens]	2.55E-06	0.90	10.18	46982.1	33504489	1
plasminogen [Homo sapiens]	2.87E-06	4.13	50.24	90510.2	4505881	5
filamin 1 (actin-binding protein-280) [Homo sapiens]	4.13E-06	0.80	10.15	280583.4	4503745	1
fibrinogen, beta chain preproprotein [Homo sapiens]	4.45E-06	0.96	10.20	55892.2	70906435	1
secretory leukocyte peptidase inhibitor precursor [Homo sapiens]	5.37E-06	0.96	10.16	14315.9	4507065	1
PDZ and LIM domain 7 isoform 1 [Homo sapiens]	6.69E-06	0.87	10.13	49812.6	11496885	1
apolipoprotein D precursor [Homo sapiens]	6.78E-06	2.01	30.18	21261.8	4502363	3
insulin-like growth factor 2 [Homo sapiens]	8.75E-06	1.84	20.15	20127.3	4504689	2
insulin-like growth factor binding protein 7 [Homo sapiens]	1.21E-05	0.95	10.20	29111.5	4504619	1
profilin 1 [Homo sapiens]	1.55E-05	1.59	20.17	15044.6	4826898	2
prostaglandin H2 D-isomerase [Homo sapiens]	2.14E-05	0.96	10.20	21015.4	32171249	1
protease, serine, 1 preproprotein [Homo sapiens]	3.33E-05	0.89	10.18	26541.1	4506145	1
heparanase [Homo sapiens]	3.71E-05	1.91	20.15	61110.2	94721347	2
coagulation factor V precursor [Homo sapiens]	3.77E-05	0.92	10.17	251559.8	4503643	1
small inducible cytokine A18 precursor [Homo sapiens]	3.93E-05	1.83	20.18	9842.1	4506831	2
cystatin C precursor [Homo sapiens]	4.21E-05	0.91	10.15	15789.1	4503307	1
ubiquitin and ribosomal protein S27a precursor [Homo sapiens]	4.40E-05	0.87	10.17	17953.5	4506713	1
ubiquitin C [Homo sapiens]	4.40E-05	0.87	10.17	76981.6	67191308	9
complement component 1, r subcomponent [Homo sapiens]	5.39E-05	0.89	10.19	80148.0	66347875	1
solute carrier family 13 member 3 isoform a [Homo sapiens]	5.91E-05	0.67	10.12	66797.6	31377715	1
ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin) [Homo sapiens]	1.34E-04	0.92	10.19	18342.1	4506549	1
multimerin 1 [Homo sapiens]	1.34E-04	2.59	30.19	138822.2	45289141	3
LIM and SH3 protein 1 [Homo sapiens]	4.58E-04	0.90	10.15	29698.2	5453710	1
serum amyloid A4, constitutive [Homo sapiens]	4.59E-04	0.94	10.16	14797.3	10835095	1
propionyl-Coenzyme A carboxylase, alpha polypeptide precursor [Homo sapiens]	5.90E-04	0.35	10.13	80088.2	65506442	1
hypothetical protein LOC285513 [Homo sapiens]	7.16E-04	0.59	10.14	82378.8	38053857	1
PDZ and LIM domain 1 (elfin) [Homo sapiens]	9.29E-04	0.82	10.12	36049.1	13994151	1
dermcidin preproprotein [Homo sapiens]	1.00E+00	0.80	10.14	11276.8	16751921	1
kininogen 1 [Homo sapiens]	1.00E+00	0.50	10.12	47852.7	4504893	1

APPLICATIONS

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



Envisioned application of particles: skin patch for biomarker harvesting from sweat

REFERENCES

1. M. Gaspari, et al., *Journal of Proteome Research*, Vol. 5, No. 5, May 2006, pp. 1261-1266.