SUPPORTING INFORMATION

Integration of protein processing steps on a droplet microfluidics platform for MALDI-MS analysis

Debalina Chatterjee,[1] A. Jimmy Ytterberg,[1] Sang Uk Son,[1] Joseph A. Loo[1,2] and Robin L. Garrell[1,3*]

[1]Department of Chemistry and Biochemistry, [2]Department of Biological Chemistry, and [3]California NanoSystems Institute, University of California Los Angeles, Los Angeles, California USA 90095-1569

*Corresponding author

E-mail: garrell@chem.ucla.edu
Tel: +1-(310) 825-2496
Fax: +1-(310) 206-2061

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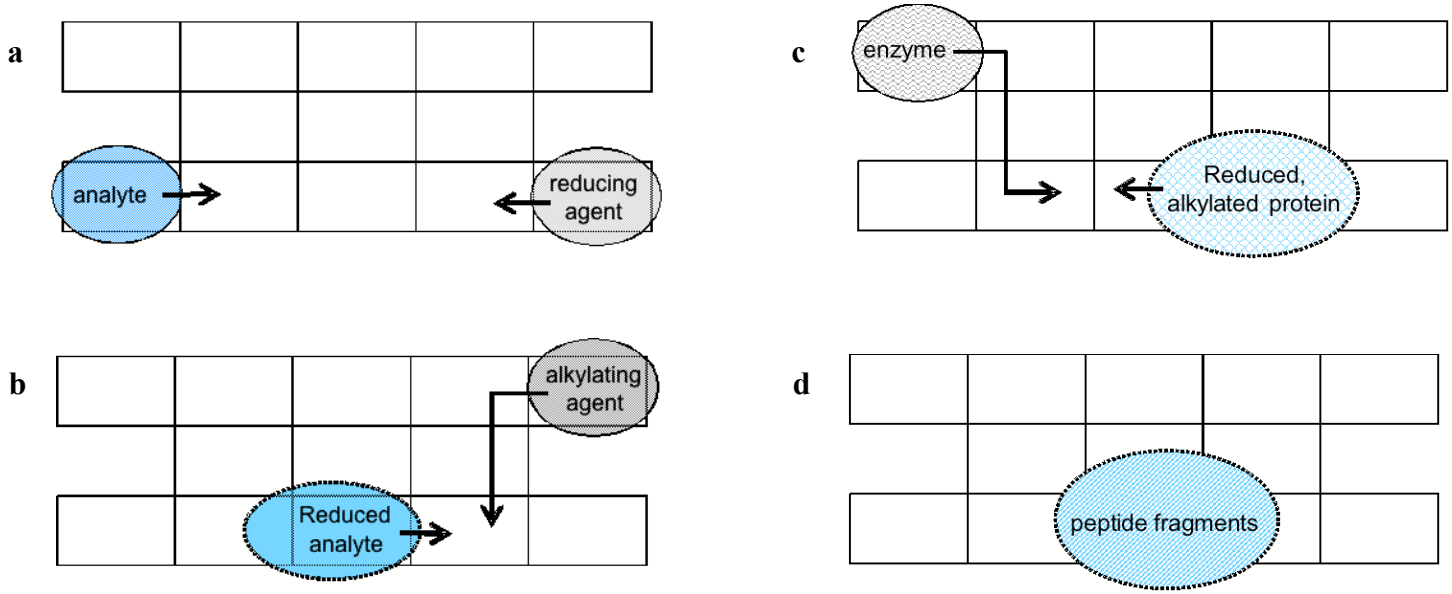
1. Protein processing steps and device operation schematic

A schematic of the protein processing steps is shown below. (a) Droplets containing the protein analyte (carbonic anhydrase, cytochrome *c*, ubiquitin, insulin, BSA or lysozyme) and reducing agent TCEP were merged and allowed to react at room temperature for 5 min. For the mixture of proteins containing BSA, lysozyme, transferrin, RNase A and α-lactalbumin, before merging with TCEP, a droplet of acetonitrile was transported on the device and merged with the protein mixture to facilitate movement. (Because acetonitrile is quite volatile, it was necessary to ensure that transport to the necessary site proceeded rapidly.) (b) After the 5 min reduction, a third droplet containing alkylating reagent NEM was transported to the reduced analyte and allowed to react for 5 min. (c) A fourth droplet containing the proteolytic enzyme trypsin was transported to the reduced, alkylated analyte and allowed to react for 15 min at room temperature. In some preliminary experiments, digestion with trypsin was allowed to proceed in an incubator (VWR 1910, VWR Scientific Inc. West Chester, PA) at 37 ˚C for 4 h. (d) Finally, a droplet containing the matrix, α-cyano or DHB, was dispensed into the processed analyte droplet and allowed to dry in air. The device was affixed to a custom MALDI target plate and inserted into the spectrometer.

When proteins are isolated and denatured, species that can interfere with the MALDI-MS analysis may be introduced. These impurities include salts, buffers, and surfactants. In our previous work, we showed that buffers and denaturing agents such as urea (up to 8 M) can be removed from protein samples by an on-chip rinsing step, and that this

step greatly improves the quality of the mass spectra subsequently obtained from the dried samples. For some samples, it might be appropriate to rinse deposited protein spot prior to reduction and alkylation. For other samples, rinsing might be needed after crystallization with the matrix; in this instance, a matrix that is insoluble in water, such as α-cyano, should be used to minimize loss of the peptide or protein analytes. In the experiments described in the main text, the samples contained no detergents or interfering buffers, so no rinsing was required.



2. Choice of alkylating agent and matrix

Alkylating agent. Three different alkylating agents commonly employed in proteomics experiments, iodoacetamide (IAA), 4-vinyl pyridine (4-VP) and *N*-ethylmaleimide (NEM) were evaluated for on-chip protein processing. IAA is light sensitive; it becomes inactive under ambient light conditions. Because our initial testing platform requires the operator to visualize droplet movement, the droplets could not easily be manipulated in the dark, and thus IAA was not tested presently. (In principle, droplet movement can be controlled without direct visualization. IAA will be tested in the future.) 4-VP is very reactive and easily polymerizes at room temperature. Droplets containing 4-VP dried as a thin film on the device surface and had to be rinsed prior to spectral acquisition. Low concentrations of 4-VP and short reaction times yielded few completely alkylated products, while higher concentrations and longer reaction times produced non-specifically alkylated products. In contrast, NEM alkylation via Michael addition was fast, easy to use, did not require manipulation of droplets in the dark or rinsing before crystallization with the MALDI matrix, and so proved to be the reagent of choice currently.

Matrix. Sinapinic acid (SA), α-cyano, and DHB were evaluated as MALDI matrix materials for co-crystallization with digests of insulin, lysozyme and BSA. When SA was used, some peptides were ionized very well, while others were not. Lysozyme and BSA peptides did not ionize well with DHB, and there was excessive background signal at lower m/z. However, both the chains of reduced insulin could be detected with DHB compared to when α-cyano was used. For all

other results reported in this paper, α-cyano was used as the matrix; this is not surprising, as α-cyano is the matrix of choice for most proteomics MALDI applications.

Adding matrix to the sample before the digestion mixture dried ensured good mixing and easier resuspension of the solvated peptides. This resulted in enhanced crystallization and hence better quality of MALDI mass spectra. Adding matrix to the dried digestion mixture resulted in poorer quality mass spectra, possibly because of incomplete matrix mixing with the resuspended/re-solvated peptides.

3. Peptide Mass Fingerprint Data for ubiquitin (bovine) and cytochrome c (bovine) (Fig 2 from paper)

Ubiquitin (bovine)
Sequence coverage: 51%
Mowse Score: 121

Start	-	End	Observed $(M+H)^+$	M_r (expt)	M_r (calc)	Error (Da)	MC[a]	Sequence
28	-	42	1722.799	1721.7917	1721.9060	-0.1143	2	K.AKIQDKEGIPPDQQR.L
30	-	42	1523.756	1522.7487	1522.7740	-0.0252	1	K.IQDKEGIPPDQQR.L
49	-	63	1779.817	1778.8097	1778.8799	-0.0701	1	K.QLEDGRTLSDYNIQK.E
49	-	72	2828.186	2827.1787	2827.4828	-0.3041	2	K.QLEDGRTLSDYNIQKESTLHLVLR.L
55	-	72	2130.071	2129.0637	2129.1480	-0.0843	1	R.TLSDYNIQKESTLHLVLR.L
64	-	72	1067.689	1066.6817	1066.6135	0.0682	0	K.ESTLHLVLR.L

[a]missed cleavage

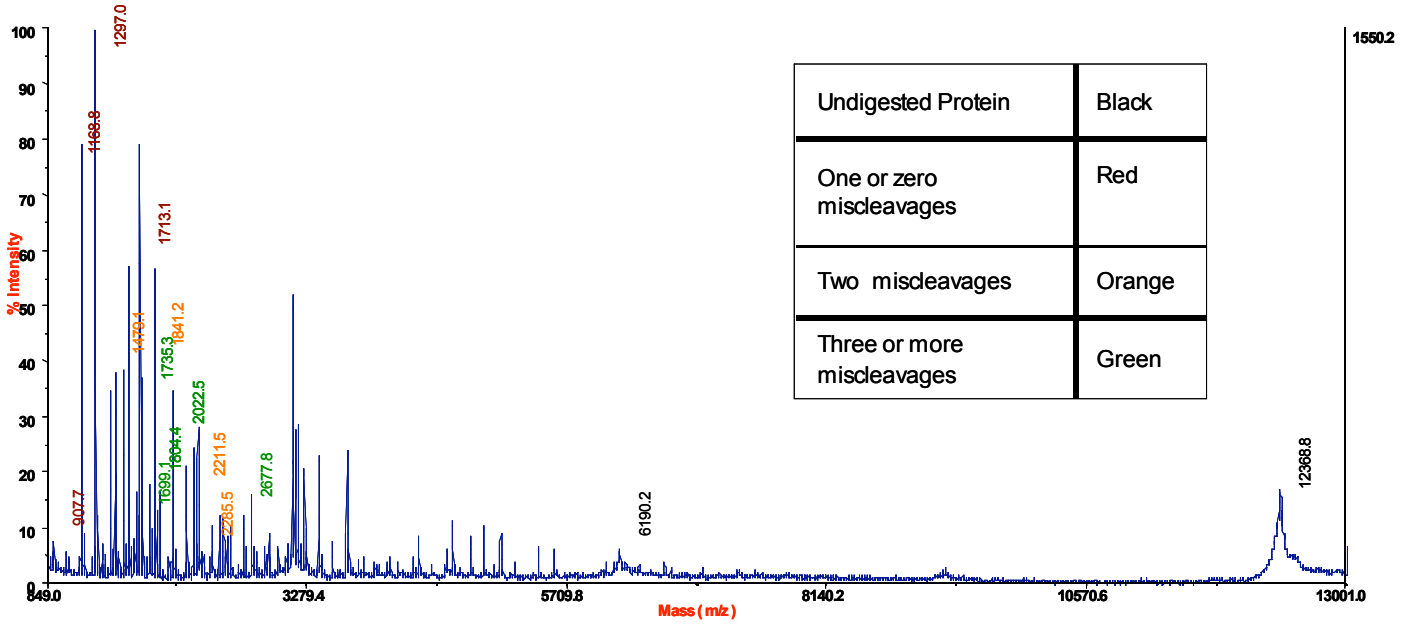
Cytochrome c (equine)
Sequence coverage: 40%
Mowse Score: 110

Start	-	End	Observed $(M+H)^+$	M_r (expt)	M_r (calc)	Error (Da)	MC[a]	Sequence
26	-	38	1433.4050	1432.3977	1432.7688	-0.3710	1	K.HKTGPNLHGLFGR.K
26	-	39	1561.6120	1560.6047	1560.8637	-0.2590	2	K.HKTGPNLHGLFGRK.T
28	-	39	1297.0770	1296.0697	1295.7099	0.3599	1	K.TGPNLHGLFGRK.T
39	-	53	1598.4580	1597.4507	1597.7736	-0.3229	1	R.KTGQAPGFTYTDANK.N
39	-	55	1840.9290	1839.9217	1839.9115	0.0102	2	R.KTGQAPGFTYTDANKNK.G
40	-	53	1470.3280	1469.3207	1469.6787	-0.3580	0	K.TGQAPGFTYTDANK.N
40	-	55	1712.6510	1711.6437	1711.8166	-0.1728	1	K.TGQAPGFTYTDANKNK.G
89	-	100	1478.4820	1477.4747	1477.8140	-0.3393	2	K.TEREDLIAYLKK.A

[a]missed cleavage

4. Peptide Mass Fingerprint spectrum for in-solution trypsin digestion of cytochrome c (bovine)

5 µM cytochrome *c* digested with 0.5 µM trypsin in 25 mM ammonium bicarbonate for 15 minutes (% total peptide sequence coverage: 55.8%; % peptide coverage 1 mis-cleavage or less: 34.6%)



5. Peptide Mass Fingerprint Data for lysozyme (chicken) and albumin (bovine) (Fig 4 from paper)

Lysozyme (chicken)
Sequence coverage: 74%
Mowse Score: 214

#	Start	-	End	Observed $(M+H)^+$	M_r (expt)	M_r (calc)	Error (ppm)	MC[a]	Sequence	Modification
2+	24	-	31	961.451	960.443	960.441	3	0	R.CELAAAMK.R	N-ethylmaleimide (C)
3-	24	-	32	992.499	991.492	991.494	-2	1	R.CELAAAMKR.H	
3+	24	-	32	1117.554	1116.547	1116.542	4	1	R.CELAAAMKR.H	N-ethylmaleimide (C)
3+ox	24	-	32	1133.529	1132.522	1132.537	-13	1	R.CELAAAMKR.H	Oxidation (M); N-ethylmaleimide (C)
4	32	-	39	1030.531	1029.524	1029.510	13	1	K.RHGLDNYR.G	
5	33	-	39	874.436	873.429	873.409	22	0	R.HGLDNYR.G	
6	33	-	51	2249.122	2248.115	2248.048	30	1	R.HGLDNYRGYSLGNWVCAAK.F	N-ethylmaleimide (C)
7-	40	-	51	1268.603	1267.595	1267.602	-5	0	R.GYSLGNWVCAAK.F	
7+	40	-	51	1393.669	1392.662	1392.650	9	0	R.GYSLGNWVCAAK.F	N-ethylmaleimide (C)
8	52	-	63	1428.673	1427.666	1427.643	16	0	K.FESNFNTQATNR.N	
9	64	-	79	1753.834	1752.826	1752.828	-1	0	R.NTDGSTDYGILQINSR.W	
10-	80	-	86	936.392	935.385	935.371	15	0	R.WWCNDGR.T	
10+	80	-	86	1061.419	1060.412	1060.419	-6	0	R.WWCNDGR.T	N-ethylmaleimide (C)
11+	80	-	91	1559.710	1558.702	1558.674	18	1	R.WWCNDGRTPGSR.N	N-ethylmaleimide (C)
12	115	-	130	1803.925	1802.918	1802.889	16	1	K.KIVSDGNGMNAWVAWR.N	
12ox	115	-	130	1819.911	1818.903	1818.884	11	1	K.KIVSDGNGMNAWVAWR.N	Oxidation (M)
13	116	-	130	1675.815	1674.808	1674.794	8	0	K.IVSDGNGMNAWVAWR.N	
13ox	116	-	130	1691.759	1690.752	1690.789	-22	0	K.IVSDGNGMNAWVAWR.N	Oxidation (M)
14-	133	-	143	1276.654	1275.646	1275.639	5	1	R.CKGTDVQAWIR.G	
14+	133	-	143	1401.702	1400.695	1400.687	6	1	R.CKGTDVQAWIR.G	N-ethylmaleimide (C)
16	135	-	143	1045.542	1044.535	1044.535	-1	0	K.GTDVQAWIR.G	

[a]missed cleavage

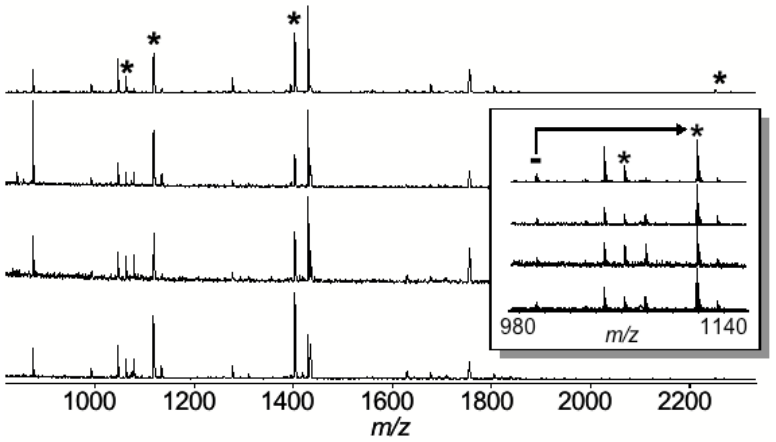
Albumin (bovine)
Sequence coverage: 37%
Mowse Score: 159

#	Start	-	End	Observed $(M+H)^+$	M_r (expt)	M_r (calc)	Error (ppm)	MC[a]	Sequence	Modification
1	25	-	34	1192.579	1192.579	1192.595	-13	1	R.DTHKSEIAHR.F	
2	35	-	44	1248.605	1248.605	1248.614	-7	1	R.FKDLGEEHFK.G	
3	66	-	75	1162.610	1162.610	1162.623	-12	0	K.LVNELTEFAK.T	
4-	89	-	105	1887.982	1887.982	1887.988	-3	1	K.SLHTLFGDELCKVASLR.E	
5	161	-	167	926.486	926.486	926.486	0	0	K.YLYEIAR.R	
6	161	-	168	1082.623	1082.623	1082.587	33	1	K.YLYEIARR.H	
7	168	-	183	2044.020	2044.020	2044.021	0	1	R.RHPYFYAPELLYYANK.Y	
8+	221	-	228	1042.531	1042.531	1042.559	-27	1	R.LRCASIQK.F	N-ethylmaleimide (C)
9	233	-	241	1000.569	1000.569	1000.582	-13	1	R.ALKAWSVAR.L	
10+	267	-	285	2451.080	2451.080	2451.014	27	1	K.ECCHGDLLECADDRADLAK.Y	3 N-ethylmaleimide (C)
11	347	-	359	1566.758	1566.758	1566.735	14	0	K.DAFLGSFLYEYSR.R	
12	360	-	371	1438.797	1438.797	1438.804	-5	1	R.RHPEYAVSVLLR.L	
13+	413	-	420	1135.440	1135.440	1135.460	-18	0	K.QNCDQFEK.L	N-ethylmaleimide (C)
14	421	-	433	1478.790	1478.790	1478.788	1	0	K.LGEYGFQNALIVR.Y	
15	437	-	451	1638.930	1638.930	1638.930	-1	1	R.KVPQVSTPTLVEVSR.S	
16	438	-	451	1510.864	1510.864	1510.836	19	0	K.VPQVSTPTLVEVSR.S	
17-	460	-	468	1176.469	1176.469	1176.490	-18	0	R.CCTKPESER.M	N-ethylmaleimide (C)
17+	460	-	468	1301.542	1301.542	1301.538	3	0	R.CCTKPESER.M	2 N-ethylmaleimide (C)
18-	469	-	482	1666.842	1666.842	1666.806	22	0	R.MPCTEDYLSLILNR.L	
18+	469	-	482	1791.848	1791.848	1791.854	-3	0	R.MPCTEDYLSLILNR.L	N-ethylmaleimide (C)
19-	499	-	507	1148.507	1148.507	1148.495	10	0	K.CCTESLVNR.R	N-ethylmaleimide (C)
19+	499	-	507	1273.521	1273.521	1273.543	-18	0	K.CCTESLVNR.R	2 N-ethylmaleimide (C)
20+	499	-	523	3203.454	3203.454	3203.473	-6	1	K.CCTESLVNRRPCFSALTPDETYVPK.A	3 N-ethylmaleimide (C)
21-	508	-	523	1822.890	1822.890	1822.892	-1	0	R.RPCFSALTPDETYVPK.A	
21+	508	-	523	1947.956	1947.956	1947.940	8	0	R.RPCFSALTPDETYVPK.A	N-ethylmaleimide(C)

[a]missed cleavage

Spectral reproducibility

To establish the reproducibility of complete on-chip sample processing sequence, multiple trials of the lysozyme analysis were performed on the same device, as well as on different devices. The MALDI-MS data collected from four trials on different devices are shown below. Peaks labeled with "-" correspond to fragments with free (–SH) cysteine residues, and those indicated with "*" correspond to fragments with NEM-alkylated cysteine residues. Inset: enlarged view for m/z between 980 and 1140. The arrow highlights one of the Cys-containing peptides and its alkylated form. In all 4 replicates, the spectra show high yield for on-chip alkylation.. The spectra are very similar, containing the same peaks and showing only moderate variations in the relative peak intensities. The %CV or co-efficient of variation was found to be 33.97 and was calculated by taking an average of the % CV's of eight different peaks



7. Effect of the surface material on analyte crystal morphology

To determine the relationship between the morphology of the crystals formed on the surface after addition of the MALDI matrix and the quality of spectra obtained, we allowed a reduced, alkylated and digested analyte, lysozyme, to crystallize on two types of surfaces: parylene and Teflon-AF. In both cases, the MALDI matrix (α-cyano) was added to the lysozyme digest before the sample dried. As the images below show, when the droplet dried on the less hydrophobic parylene, the sample droplets were more spread out and evaporated faster, generating small, homogenous, uniformly distributed crystals (left). On the more hydrophobic Teflon surface (right), the footprint shrank, concentrating the sample into a small area and yielding large, densely populated crystals. High quality spectra could only be obtained from a few "sweet spots" in these crystal mats. Spectra could more easily be obtained from most crystals on the parylene surface. We therefore incorporated parylene areas in the device to serve as the crystallization and analysis sites. We used Teflon-AF coatings elsewhere, as droplet movement is more facile on this more hydrophobic material.

Crystals of the digested analyte formed on parylene (left) and Teflon-AF (right). White area represents crystals; scale bar = 0.5 mm.