Supporting Information

Linear and Differential Ion Mobility Separations of Middle-Down Proteoforms

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Contents

Figure S3. TWIMS spectra for selected variant mixtures measured in N_2 with solvent (i) using the traveling wave speeds of 650, 1000, and 1900 m/s.

Figure S4. Pairwise linear correlations between transit times for ac and me3 variants at the traveling wave speeds of 650 and 1000 m/s. Variants with only three data points and p variants with partial data at s = 1000 m/s are not included.

Figure S5. TWIMS spectra measured with different ESI solvents: (a) four me3 variants (z = 9) with solvents (i, ii, iii), (b) K23me3 (z = 8 and 9) with solvents (i, iv).

Figure S6. TWIMS spectra for four me3 variants in z = 12 measured in N_2 using solvent (ii) and s = 650 m/s. No signal at 12+ was found for the K4me3 variant.

Figure S7. TIMS spectra (on the cross section scale) measured for exemplary me3, ac, p, and me variant species using (a) t_{ramp} = 100 - 500 ms from MeOH/H_2O solutions, (b) from MeOH/H_2O and aqueous solutions.

Figure S8. TIMS spectra for selected mixtures of p variants and traces for the two components.

Figure S9. Linear correlations between transit times for me3, ac, and p variants across charge state pairs (major peaks from Fig. 1). Variants for me (with three data points) are not included.

Figure S10. Linear correlations between separation parameters in TIMS for the acetylated and phosphorylated peptide variants in different charge states.

Figure S11. Linear correlations between separation parameters in Synapt and TIMS for the

acetylated and phosphorylated peptide variants.

Figure S12. Linear correlations between FAIMS and TIMS (left column) or TWIMS (right column) separations for (a) acetylated and (b) phosphorylated variants, r^2 marked. To facilitate comparison, the left column in (a) repeats the data from Fig. 6 in the text.

Figure S13. Linear correlations between FAIMS and TIMS (left) or TWIMS (right) separations for same acetylated (a) and phosphorylated (b) variants with different charge states selected in the two dimensions (r^2 marked).

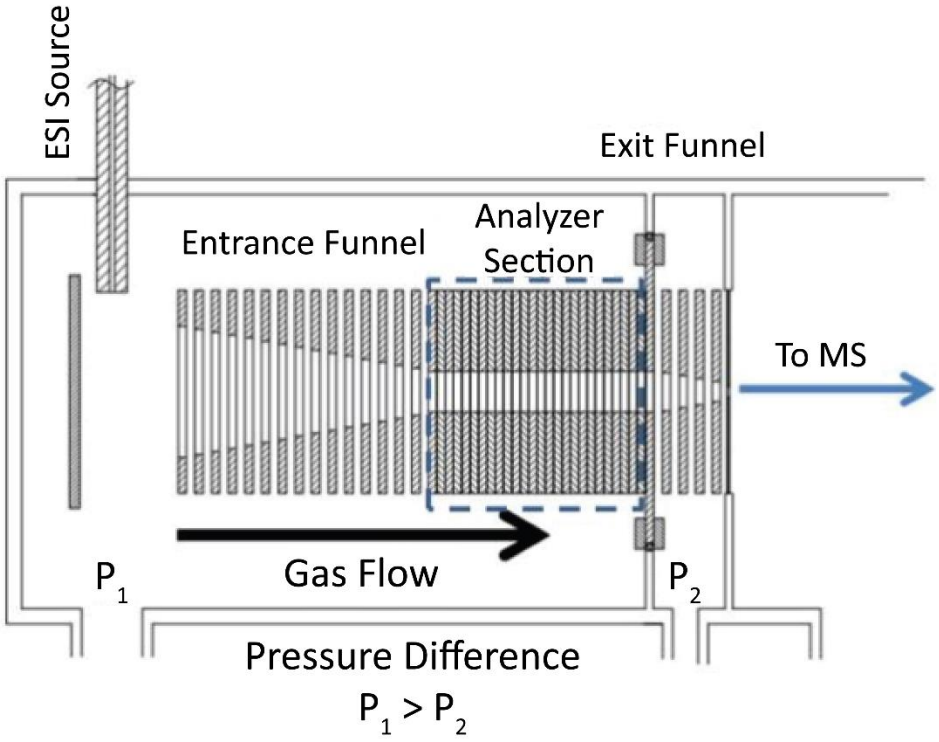
Details of nESI/TIMS hardware and mobility calibration

Nano-ESI source

Custom nESI emitters were made from quartz glass capillaries (I.D. 0.7 mm, O.D. 1.0 mm) utilizing a P-2000 micropipette laser puller (Sutter Instruments, Novato, CA). The inlet capillary was a stainless steel tube (1/16 × 0.020″, IDEX Health Science, Oak Harbor, WA).

TIMS cell

The TIMS cell is an electrodynamic ion funnel with three regions (see scheme below): entrance, analyzer tunnel (46 mm length), and exit. The rf voltage creates a quadrupolar field in the tunnel and dipolar field in other regions.



Mobility calibration in TIMS

Multiple ion species are first trapped and thermalized at different E values set up by voltage profile along the tunnel, then pushed out by "ramping" (decreasing) E in steps with each species eluting at a characteristic voltage ($V_{elution}$ - V_{out}). The total time an ion spends in the system (from gating into the TIMS cell to registration at the ToF detector) is:

$$t_{total} = t_{trap} + (V_{elution}/V_{ramp}) \times t_{ramp} + t_{ToF} = t_0 + (V_{elution}/V_{ramp}) \times t_{ramp,}$$

where t_{trap} is the trapping time, t_{ToF} is the flight time from cell exit to detector (through MS vacuum), V_{ramp} and t_{ramp} are the voltage range and time of ramp, and t_0 is the constant time before and after the separation step. The $V_{elution}$ and t_0 values for each *m/z* of interest are determined simultaneously by varying t_{ramp} from 100 to 500 ms at fixed V_{ramp}. More details on this procedure are found in Ref. [50].

Table S1.

Collision cross sections (CCS, Å²) and resolving power (*R*) values for protonated histone tails measured using TIMS.

Peptide	Ion	CCS (Å²), std. error of mean: ± 0.04%	*R*
K9me3	$[M+6H]^{6+}$	1303	137
	$[M+7H]^{7+}$	1398/1406	203/180
	$[M+8H]^{8+}$	1574/1607	96/102
	$[M+9H]^{9+}$	1685/1710/1735/1749	153/119/141/211
	$[M+10H]^{10+}$	1835	170
	$[M+11H]^{11+}$	1921	201
K23me3	$[M+6H]^{6+}$	1308	92
	$[M+7H]^{7+}$	1405/1437	82/72
	$[M+8H]^{8+}$	1508/1535/1574/1609	188/284/146/223
	$[M+9H]^{9+}$	1684/1707/1751	167/76/233
	$[M+10H]^{10+}$	1830/1839	218/236
	$[M+11H]^{11+}$	1926/1943	244/183
K36me3	$[M+6H]^{6+}$	1291/1357	128/137
	$[M+7H]^{7+}$	1394/1422/1441	59/133/119
	$[M+8H]^{8+}$	1483/1510	215/265
	$[M+9H]^{9+}$	1658/1702	240/122
	$[M+10H]^{10+}$	1833	176
	$[M+11H]^{11+}$	1948	229
K9ac	$[M+6H]^{6+}$	1209	105
	$[M+7H]^{7+}$	1290	145
	$[M+8H]^{8+}$	1395/1404/1420/1435/1447/1473	199/187/202/144/90/184
	$[M+9H]^{9+}$	1529/1563/1572/1603	191/223/197/255
	$[M+10H]^{10+}$	1673/1694/1715	222/121/159
	$[M+11H]^{11+}$	1784	133
K14ac	$[M+6H]^{6+}$	1205	120
	$[M+7H]^{7+}$	1298/1322	137/85
	$[M+8H]^{8+}$	1404/1411/1427/1434/1442/1451	134/168/153/163/170/45
	$[M+9H]^{9+}$	1541/1562/1572/1587/1625/1640	220/112/210/122/216/262
	$[M+10H]^{10+}$	1701	150
	$[M+11H]^{11+}$	1785/1814	223/151
K18ac	$[M+6H]^{6+}$	1207	72
	$[M+7H]^{7+}$	1287/1310	239/218
	$[M+8H]^{8+}$	1396/1405/1418/1428/1435/1454/1480	208/156/153/111/197/117/159
	$[M+9H]^{9+}$	1531/1563/1579/1611	247/223/198/201
	$[M+10H]^{10+}$	1694	179
	$[M+11H]^{11+}$	1786	192
K27ac	$[M+6H]^{6+}$	1210	145
	$[M+7H]^{7+}$	1294/1304/1326	223/215/221

K36ac	$[M+8H]^{8+}$	1412/1420/1438/1452/1459/1484/1498	157/144/150/190/131/206/156
	$[M+9H]^{9+}$	1558/1572/1616	180/194/171
	$[M+10H]^{10+}$	1695	131
	$[M+11H]^{11+}$	1789	145
T3p	$[M+6H]^{6+}$	1217	124
	$[M+7H]^{7+}$	1294/1301/1315	193/239/198
	$[M+8H]^{8+}$	1373/1396/1406/1425/1444/1456/1475	177/47/201/88/79/209/88
	$[M+9H]^{9+}$	1526/1555/1575/1605/1621	209/216/108/189/80
	$[M+10H]^{10+}$	1683	155
	$[M+11H]^{11+}$	1770/1792	197/201
T6p	$[M+6H]^{6+}$	1305	161
	$[M+7H]^{7+}$	1386/1412	75/150
	$[M+8H]^{8+}$	1556/1571/1596	134/118/114
	$[M+9H]^{9+}$	1653/1691/1734	221/72/73
	$[M+10H]^{10+}$	1786	194
	$[M+11H]^{11+}$	1878	146
S10p	$[M+6H]^{6+}$	1319	134
	$[M+7H]^{7+}$	1412	159
	$[M+8H]^{8+}$	1500/1523/1532/1568/1581/1612	70/206/174/204/144/269
	$[M+9H]^{9+}$	1669/1714/1749	121/163/192
	$[M+10H]^{10+}$	1805	160
	$[M+11H]^{11+}$	1921	209
S28p	$[M+6H]^{6+}$	1300	122
	$[M+7H]^{7+}$	1401/1414/1425	246/267/285
	$[M+8H]^{8+}$	1511/1520/1534/1553/1565/1582/1593/1611	232/179/197/219/142/115/306/298
	$[M+9H]^{9+}$	1665/1706/1736/1753/1767	86/74/235/428/89
	$[M+10H]^{10+}$	1783/1800/1852	244/243/197
	$[M+11H]^{11+}$	1917	167
Y41p	$[M+6H]^{6+}$	1307/1327	82/140
	$[M+7H]^{7+}$	1380/1396/1423	88/170/169
	$[M+8H]^{8+}$	1515/1554/1579	205/187/106
	$[M+9H]^{9+}$	1695/1726	138/149
	$[M+10H]^{10+}$	1829	116
	$[M+11H]^{11+}$	1924	163
K4me	$[M+6H]^{6+}$	1270	155
	$[M+7H]^{7+}$	1362/1379	91/136
	$[M+8H]^{8+}$	1464/1499/1529/1553	229/259/118/174
	$[M+9H]^{9+}$	1627/1691/1659/1744	258/148/210/208
	$[M+10H]^{10+}$	1824	182
	$[M+11H]^{11+}$	1922/1869	180/228
	$[M+6H]^{6+}$	1218	129
	$[M+7H]^{7+}$	1303/1315/1326	200/146/199
	$[M+8H]^{8+}$	1414/1428/1439/1452/1465/1493/1506/1517	89/291/175/278/144/138/232/124

K9me	$[M+9H]^{9+}$	1627/1638/1651	148/252/229
	$[M+10H]^{10+}$	1695/1730/1750	213/122/221
	$[M+11H]^{11+}$	1791/1799	167/189
K23me	$[M+6H]^{6+}$	1212	135
	$[M+7H]^{7+}$	1301/1324	190/221
	$[M+8H]^{8+}$	1426/1438/1473/1499/1511	247/173/142/180/140
	$[M+9H]^{9+}$	1574/1591/1628/1640	237/175/119/149
	$[M+10H]^{10+}$	1704	97
	$[M+11H]^{11+}$	1787	167
	$[M+6H]^{6+}$	1218	151
	$[M+7H]^{7+}$	1302/1309/1326/1336	193/73/165/69
	$[M+8H]^{8+}$	1416/1430/1439/1453/1468/1494/1507	153/164/212/153/120/199/260
	$[M+9H]^{9+}$	1561/1576/1625/1635	244/146/140/178
	$[M+10H]^{10+}$	1700	90
	$[M+11H]^{11+}$	1800	143

Figure S1

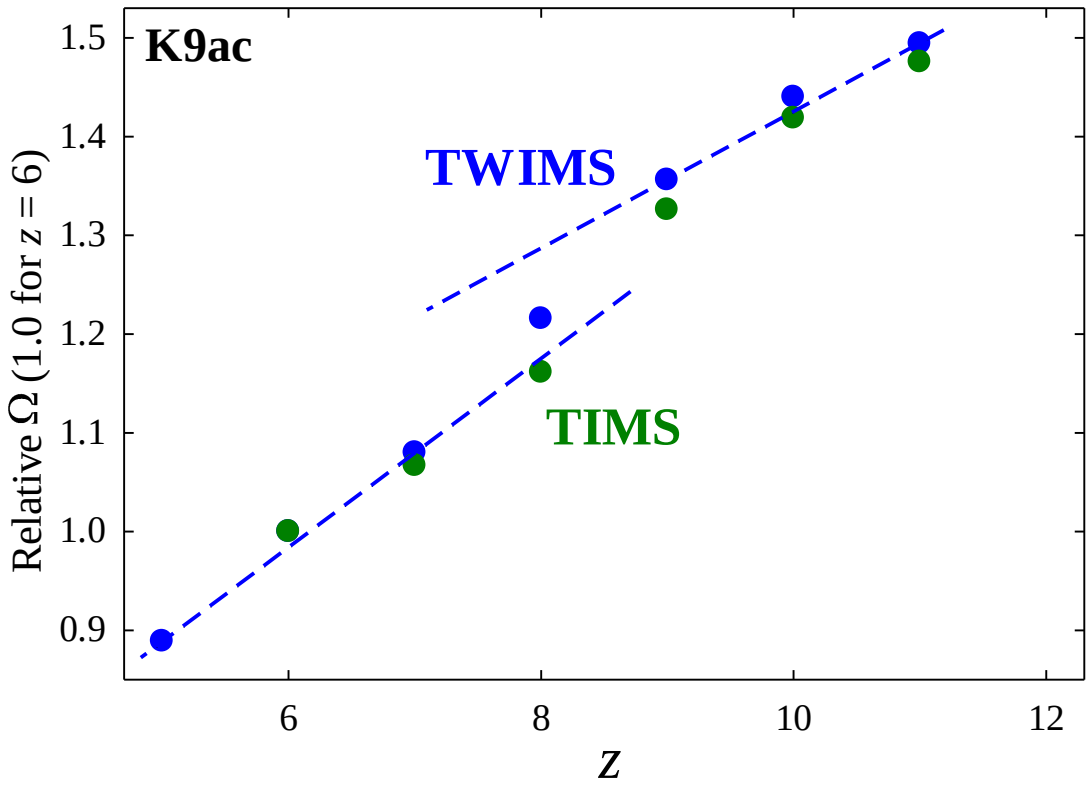

K9ac
TWIMS
TIMS
Relative Ω (1.0 for z = 6)
z
1.5
1.4
1.3
1.2
1.1
1.0
0.9
6
8
10
12

Figure S2

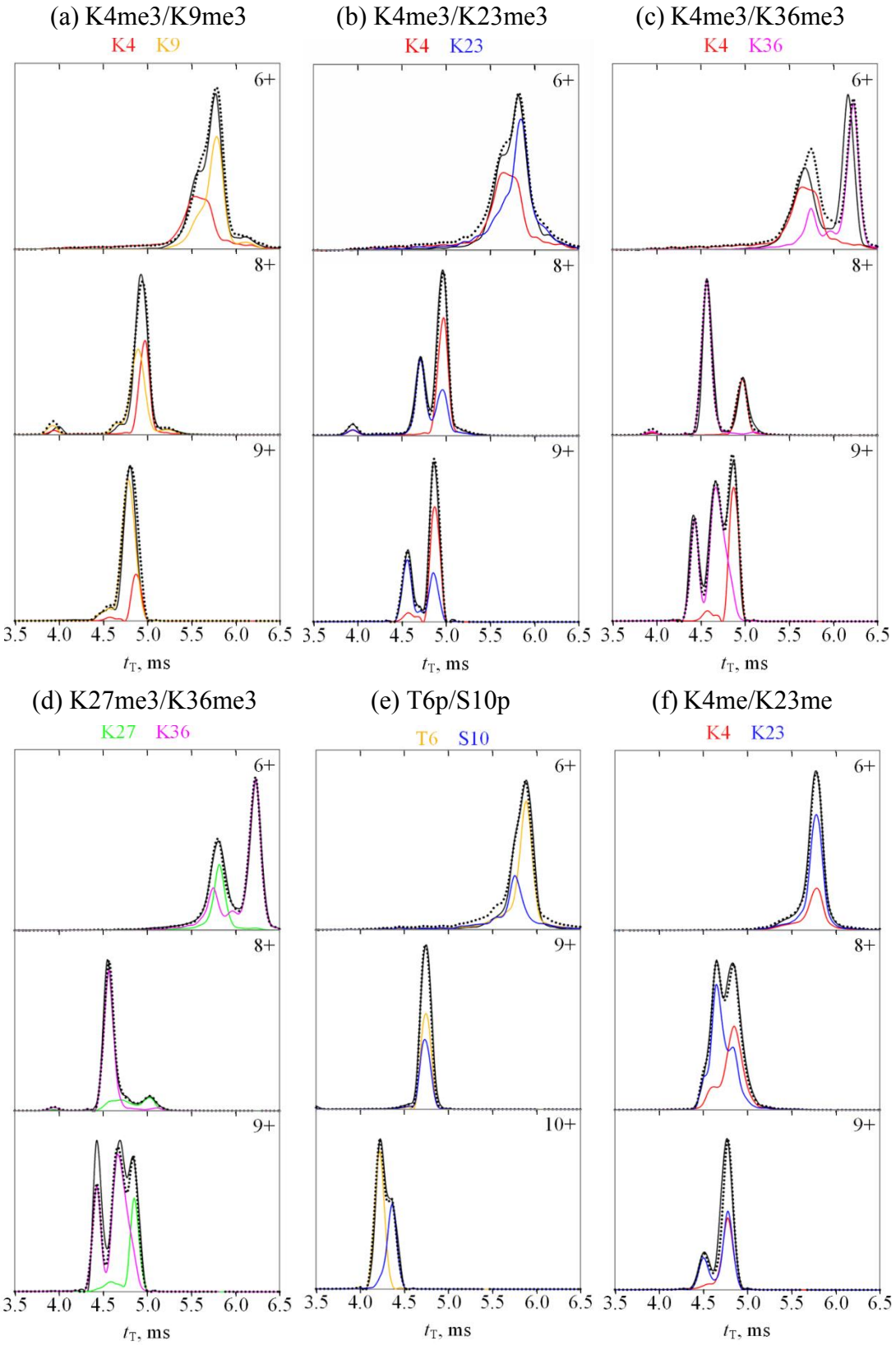

(a) K4me3/K9me3
K4 K9
(b) K4me3/K23me3
K4 K23
(c) K4me3/K36me3
K4 K36
(d) K27me3/K36me3
K27 K36
(e) T6p/S10p
T6 S10
(f) K4me/K23me
K4 K23
6+
8+
9+
10+
3.5 4.0 4.5 5.0 5.5 6.0 6.5
t_T, ms

Figure S3

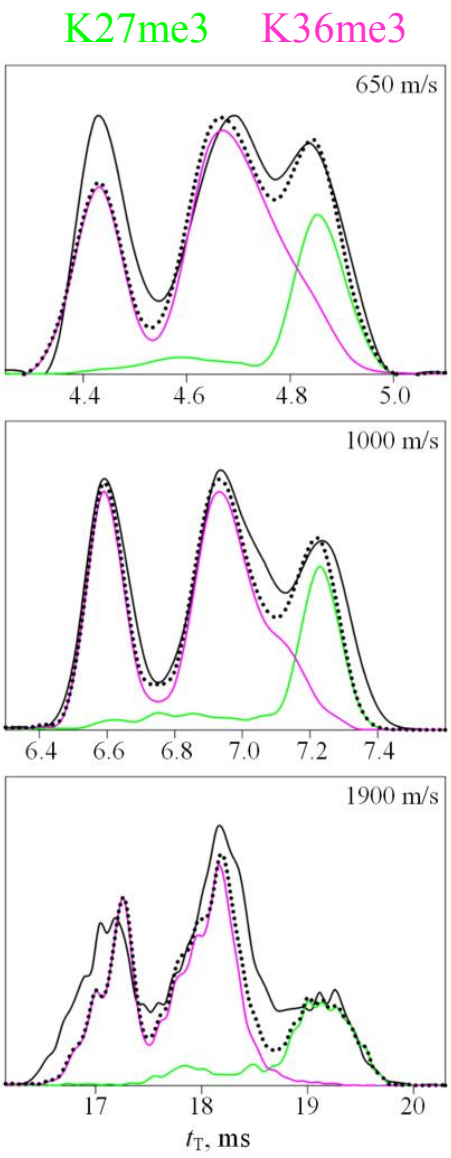

K27me3
K36me3
650 m/s
4.4
4.6
4.8
5.0
1000 m/s
6.4
6.6
6.8
7.0
7.2
7.4
1900 m/s
17
18
19
20
t_T, ms

Figure S3 (continued)

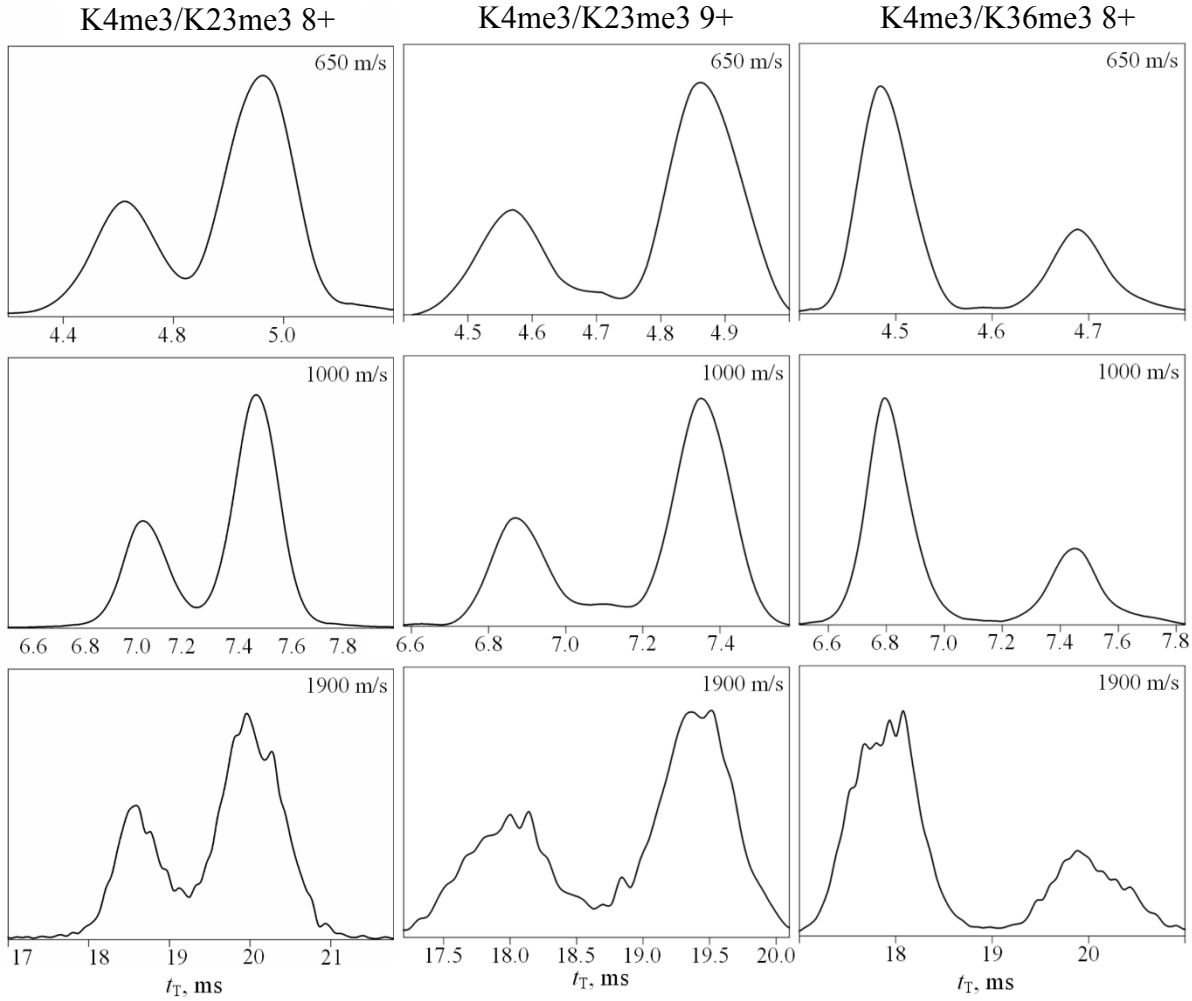


Figure S3 (continued)

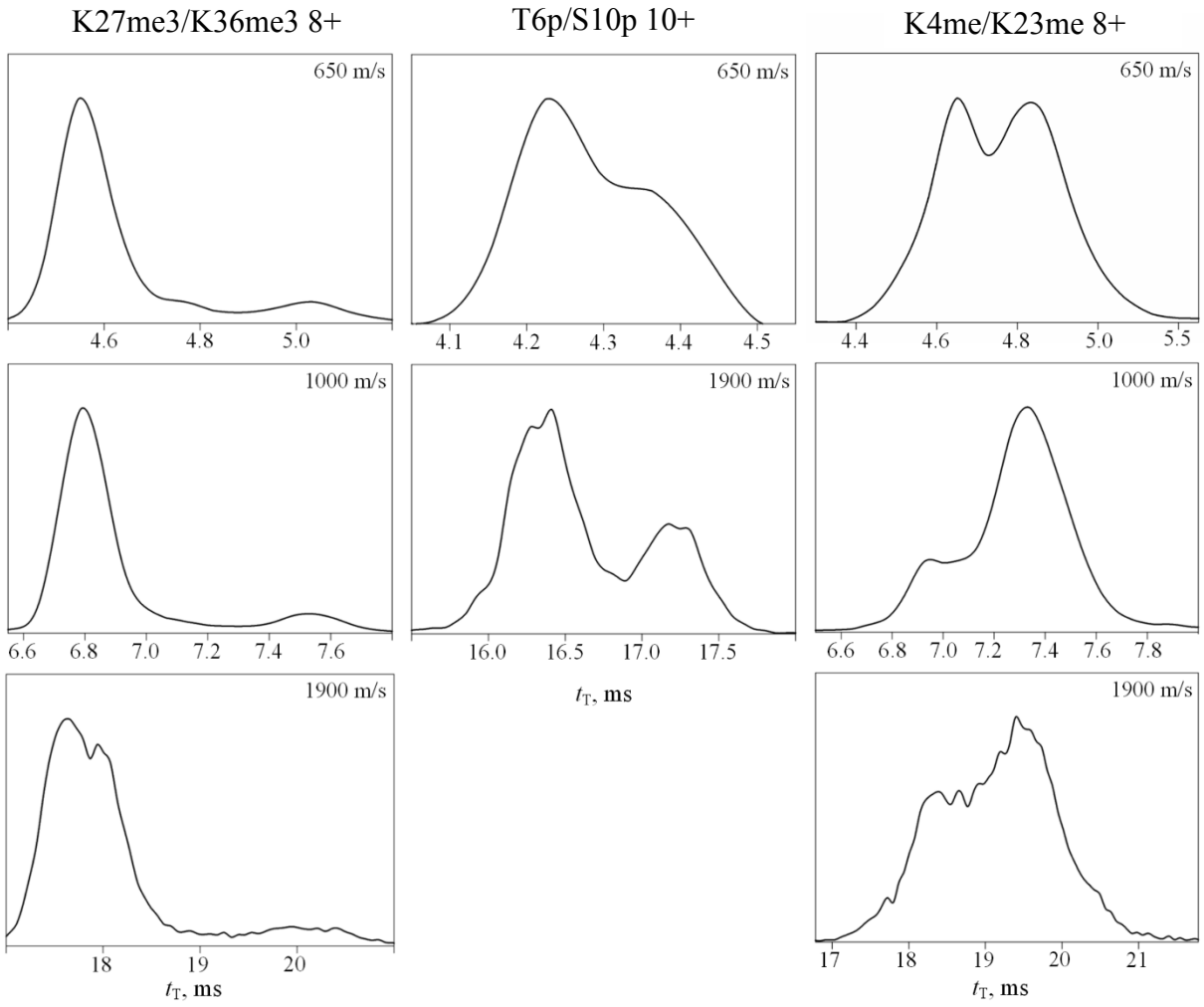


Figure S4

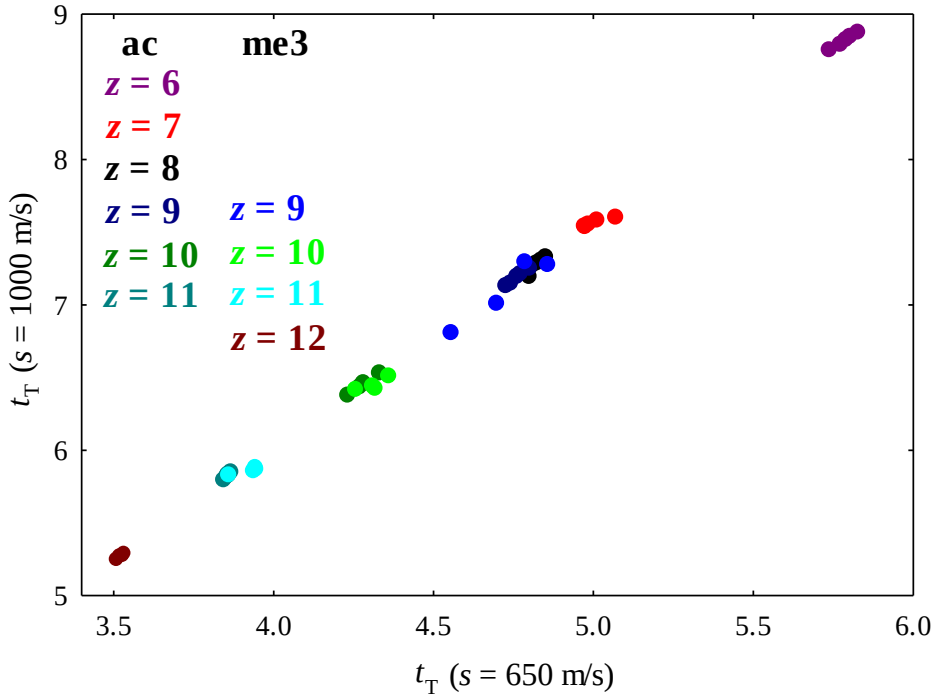

ac
me3
z = 6
z = 7
z = 8
z = 9
z = 10
z = 11
z = 9
z = 10
z = 11
z = 12
9
8
7
6
5
3.5
4.0
4.5
5.0
5.5
6.0
t_T (s = 1000 m/s)
t_T (s = 650 m/s)

Figure S5

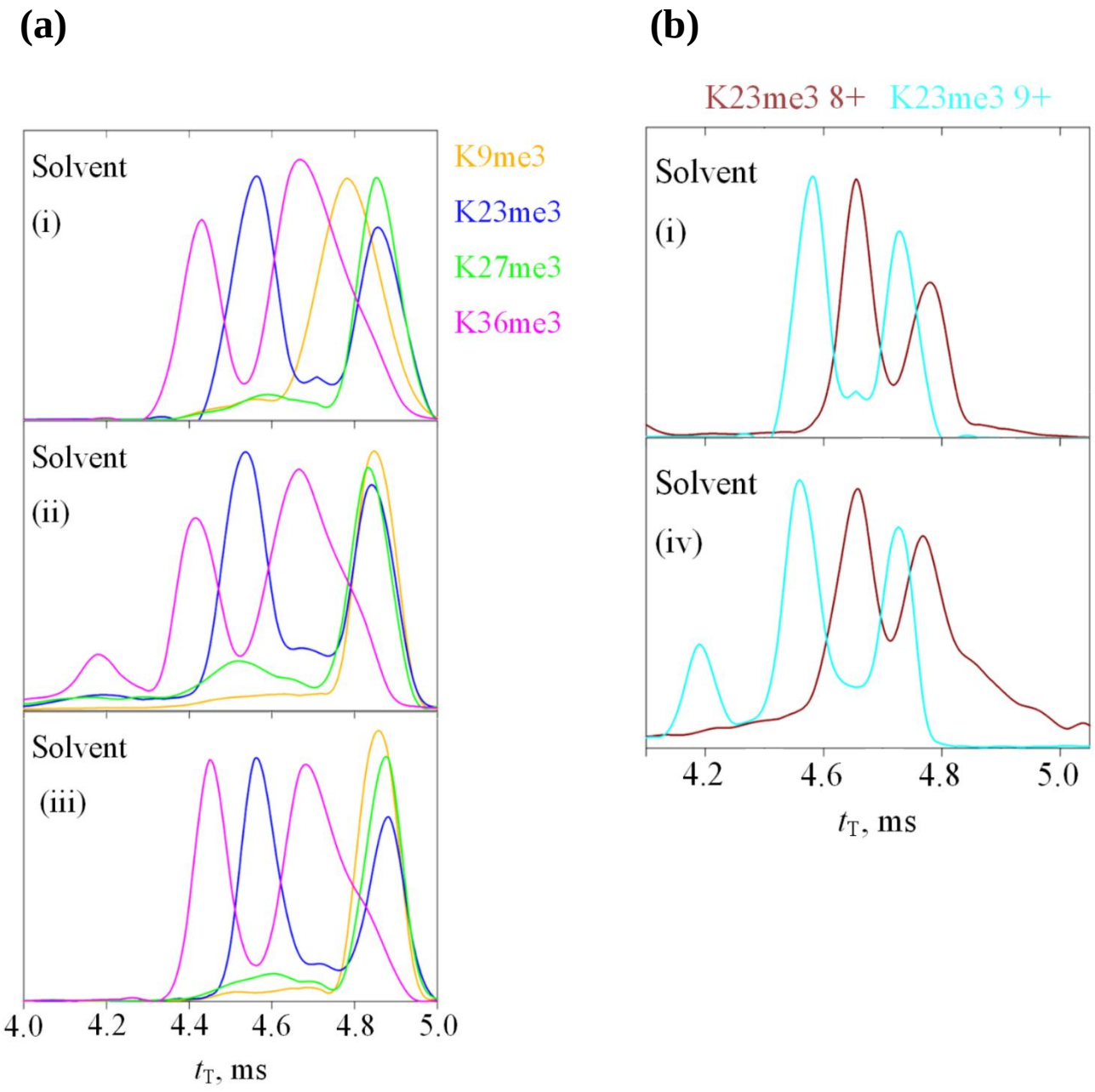

(a)
(b)
Solvent
(i)
Solvent
(ii)
Solvent
(iii)
K9me3
K23me3
K27me3
K36me3
4.0
4.2
4.4
4.6
4.8
5.0
t_T, ms
K23me3 8+
K23me3 9+
Solvent
(i)
Solvent
(iv)
4.2
4.6
4.8
5.0
t_T, ms

Figure S6

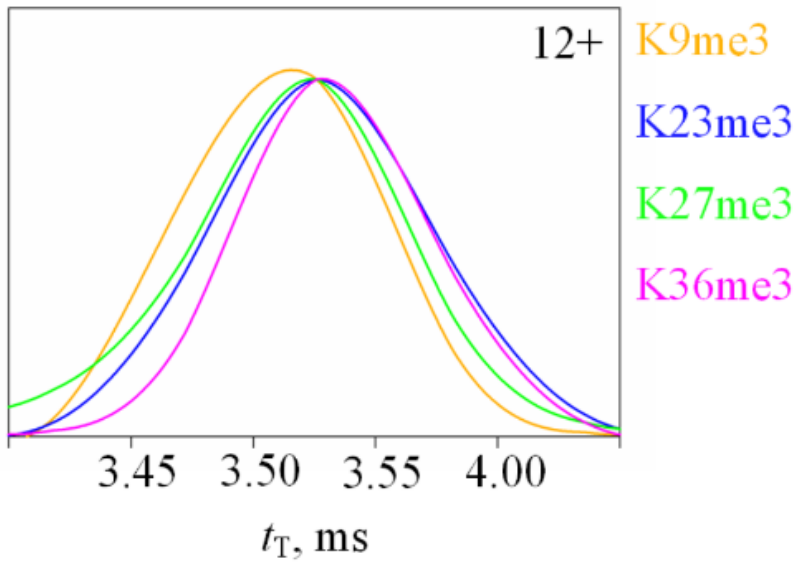
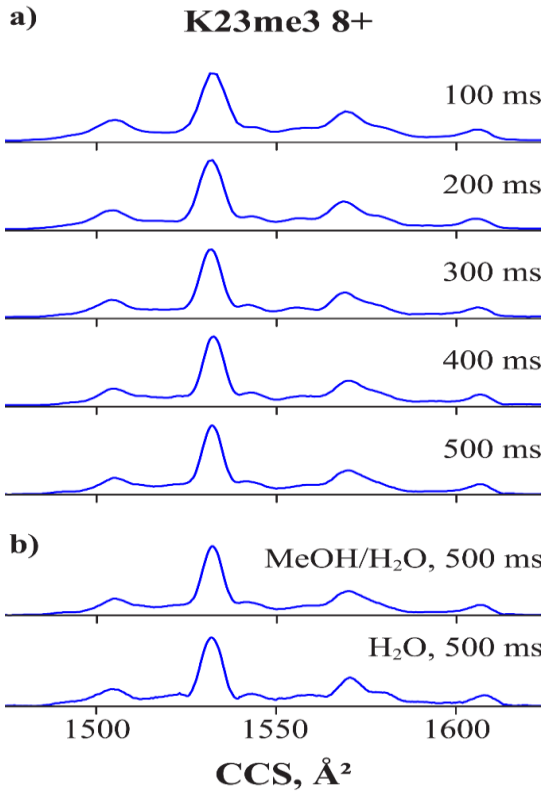


Figure S7


a)
K23me3 8+
100 ms
200 ms
300 ms
400 ms
500 ms
b)
MeOH/H2O, 500 ms
H2O, 500 ms
1500
1550
1600
CCS, Å²

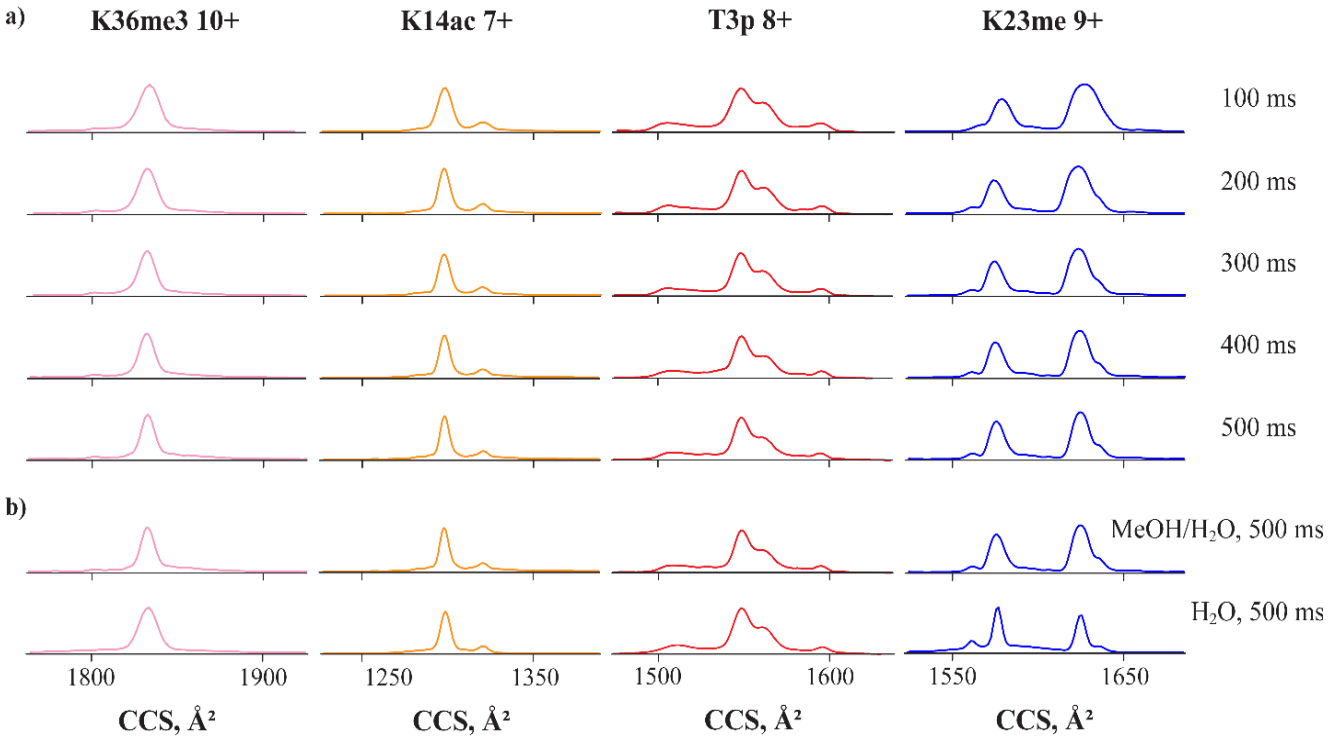

a)
K36me3 10+
K14ac 7+
T3p 8+
K23me 9+
100 ms
200 ms
300 ms
400 ms
500 ms
b)
MeOH/H2O, 500 ms
H2O, 500 ms
1800
1900
1250
1350
1500
1600
1550
1650
CCS, Å²
CCS, Å²
CCS, Å²
CCS, Å²

Figure S8

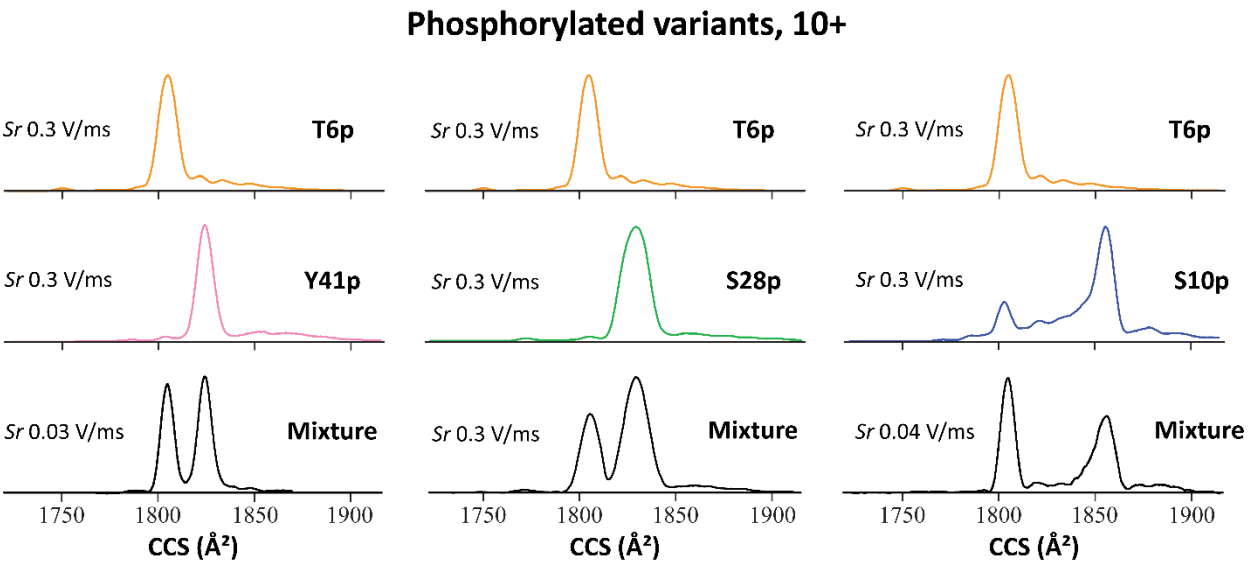

Phosphorylated variants, 10+
Sr 0.3 V/ms
T6p
Sr 0.3 V/ms
T6p
Sr 0.3 V/ms
T6p
Sr 0.3 V/ms
Y41p
Sr 0.3 V/ms
S28p
Sr 0.3 V/ms
S10p
Sr 0.03 V/ms
Mixture
Sr 0.3 V/ms
Mixture
Sr 0.04 V/ms
Mixture
1750
1800
1850
1900
CCS (Å²)
1750
1800
1850
1900
CCS (Å²)
1750
1800
1850
1900
CCS (Å²)

Figure S9

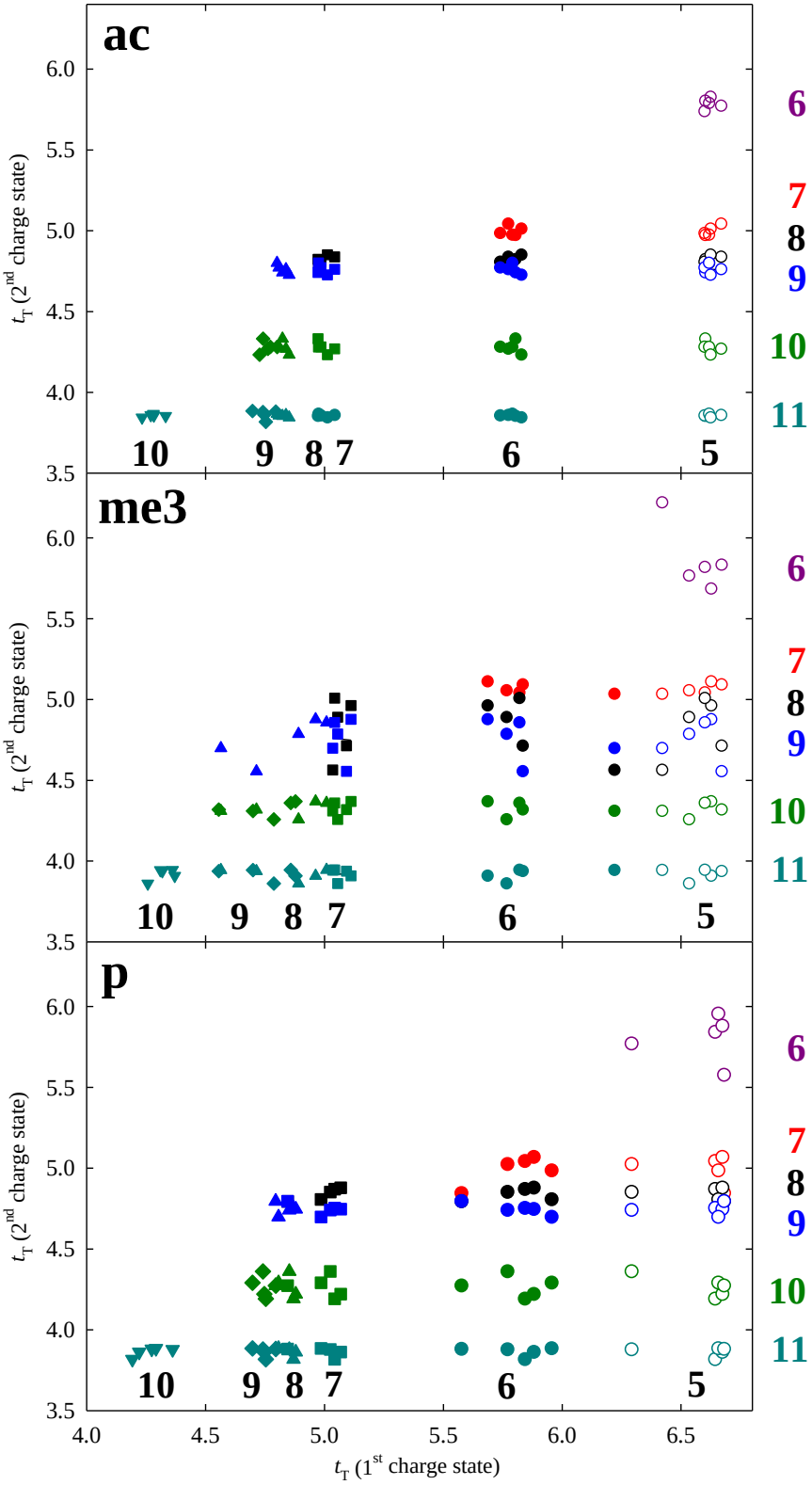
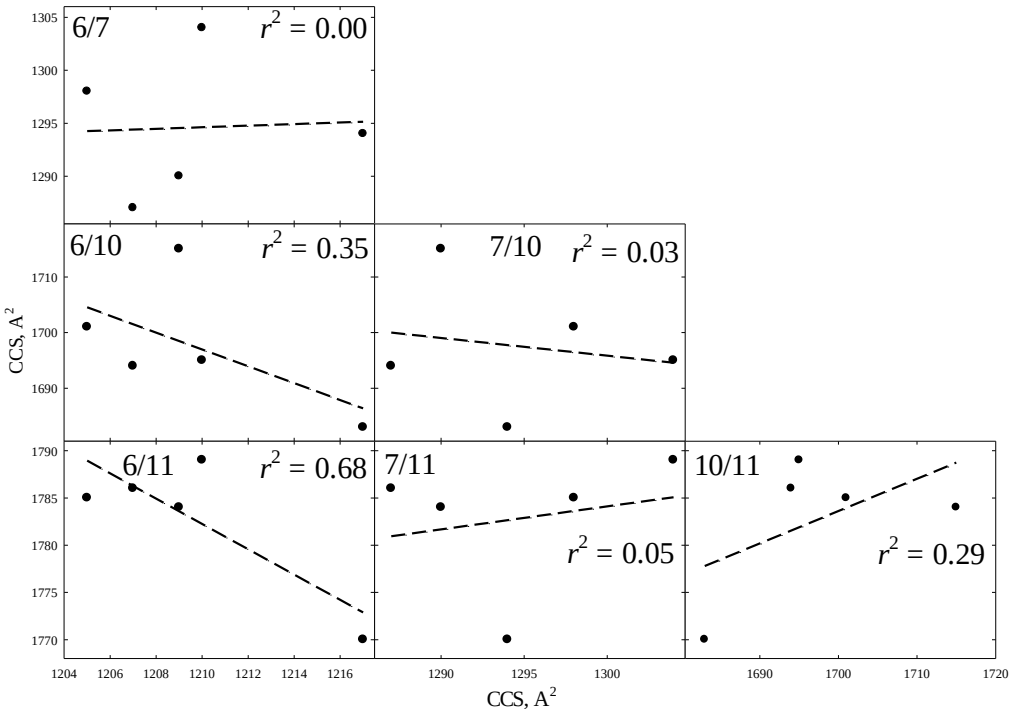

ac
me3
p
6
7
8
9
10
11
10
9
8
7
6
5
t_T (2nd charge state)
t_T (1st charge state)
6.0
5.5
5.0
4.5
4.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5

Figure S10

Acetylated



Phosphorylated

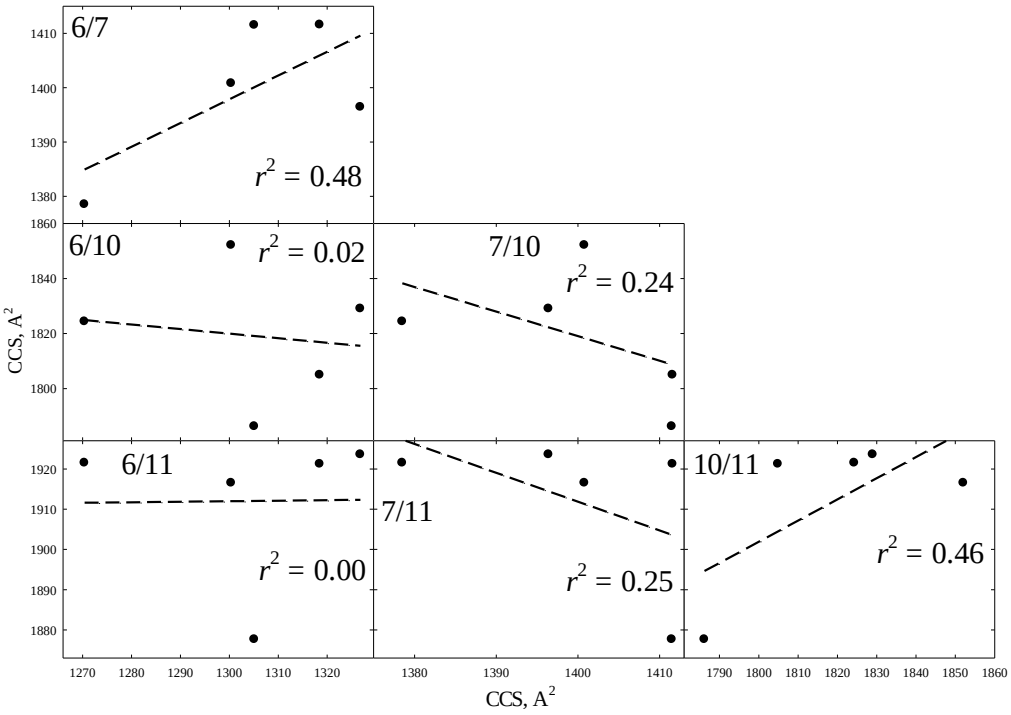


Figure S11

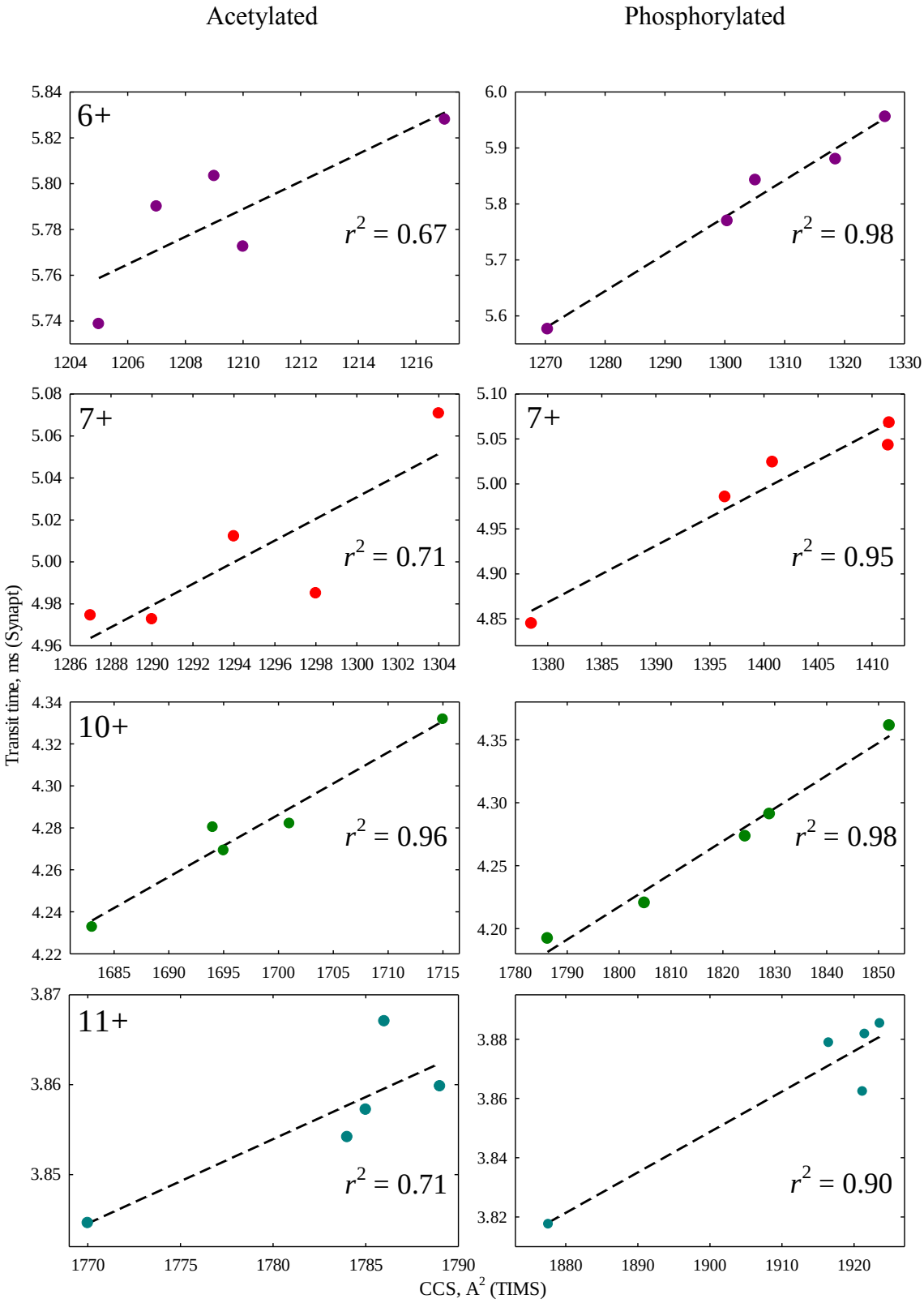

Acetylated
Phosphorylated
6+
r^2 = 0.67
r^2 = 0.98
7+
r^2 = 0.71
7+
r^2 = 0.95
10+
r^2 = 0.96
r^2 = 0.98
11+
r^2 = 0.71
r^2 = 0.90
Transit time, ms (Synapt)
CCS, A^2 (TIMS)

Figure S12a

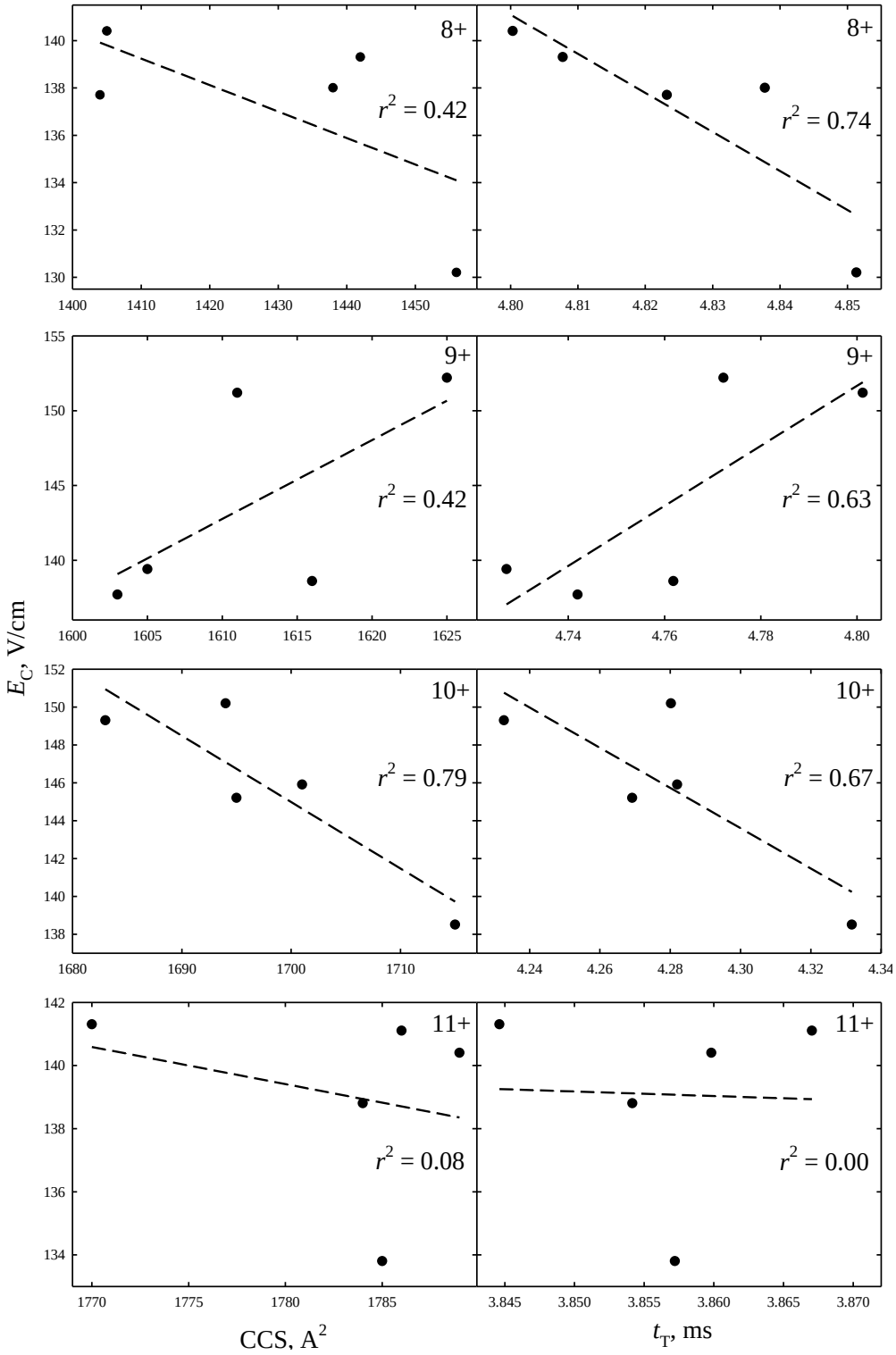

8+
r^2 = 0.42
8+
r^2 = 0.74
9+
r^2 = 0.42
9+
r^2 = 0.63
10+
r^2 = 0.79
10+
r^2 = 0.67
11+
r^2 = 0.08
11+
r^2 = 0.00
E_C, V/cm
CCS, A^2
t_T, ms

Figure S12b

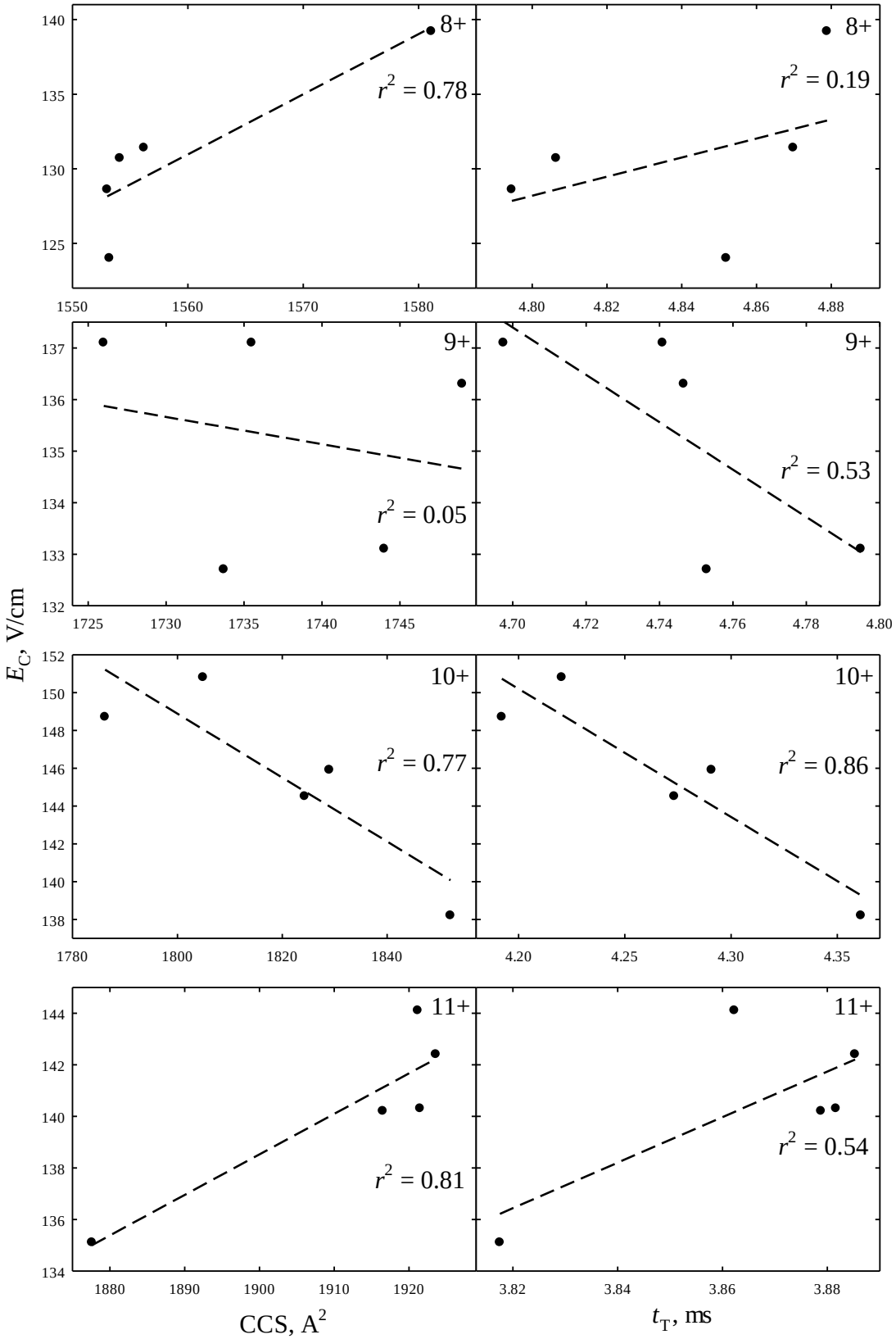

8+
r^2 = 0.78
8+
r^2 = 0.19
9+
r^2 = 0.05
9+
r^2 = 0.53
10+
r^2 = 0.77
10+
r^2 = 0.86
11+
r^2 = 0.81
11+
r^2 = 0.54
E_C, V/cm
CCS, A^2
t_T, ms

Figure S13a

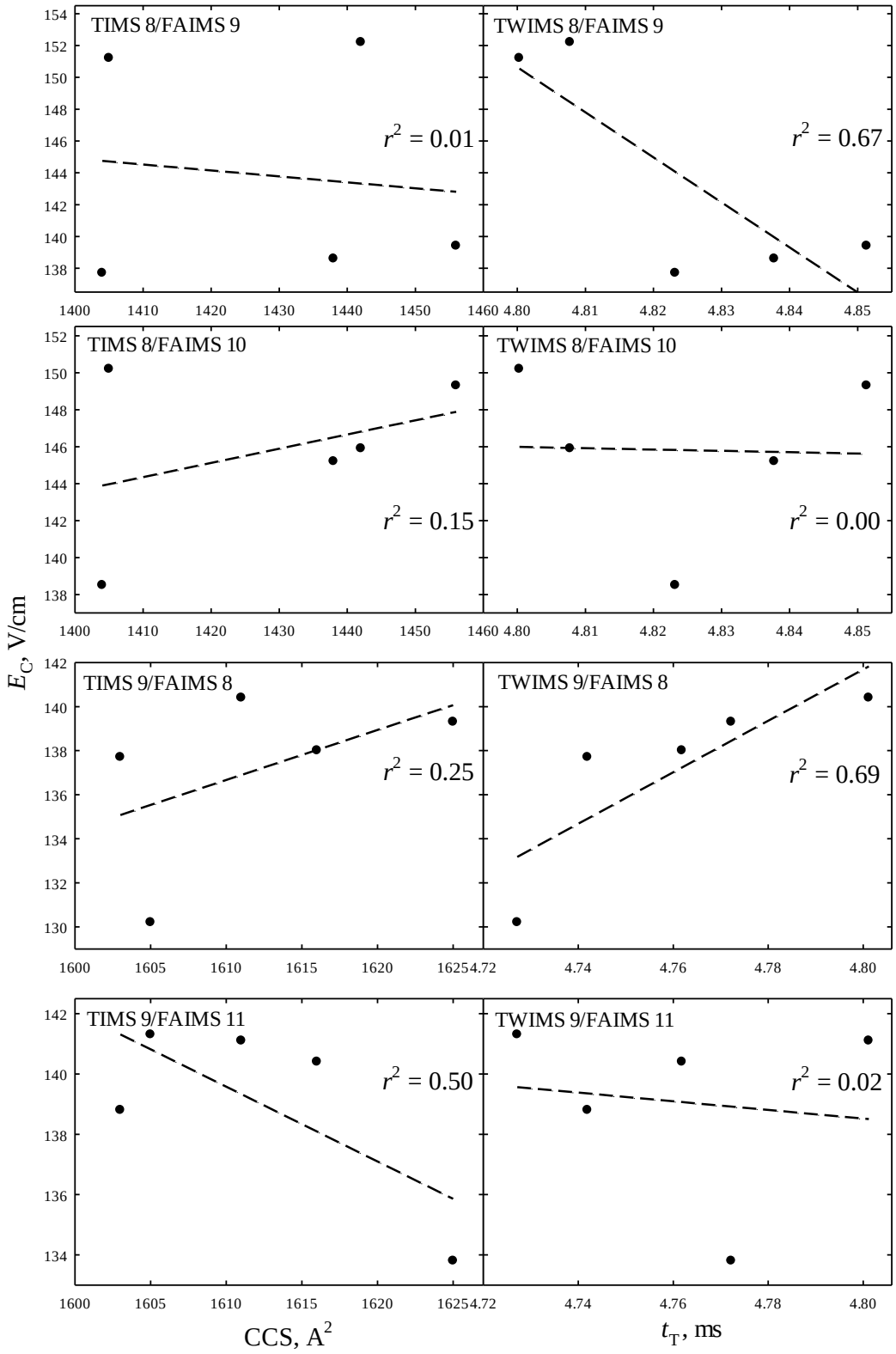

TIMS 8/FAIMS 9
r^2 = 0.01
TWIMS 8/FAIMS 9
r^2 = 0.67
TIMS 8/FAIMS 10
r^2 = 0.15
TWIMS 8/FAIMS 10
r^2 = 0.00
TIMS 9/FAIMS 8
r^2 = 0.25
TWIMS 9/FAIMS 8
r^2 = 0.69
TIMS 9/FAIMS 11
r^2 = 0.50
TWIMS 9/FAIMS 11
r^2 = 0.02
E_C, V/cm
CCS, A^2
t_T, ms

Figure S13a (continued)

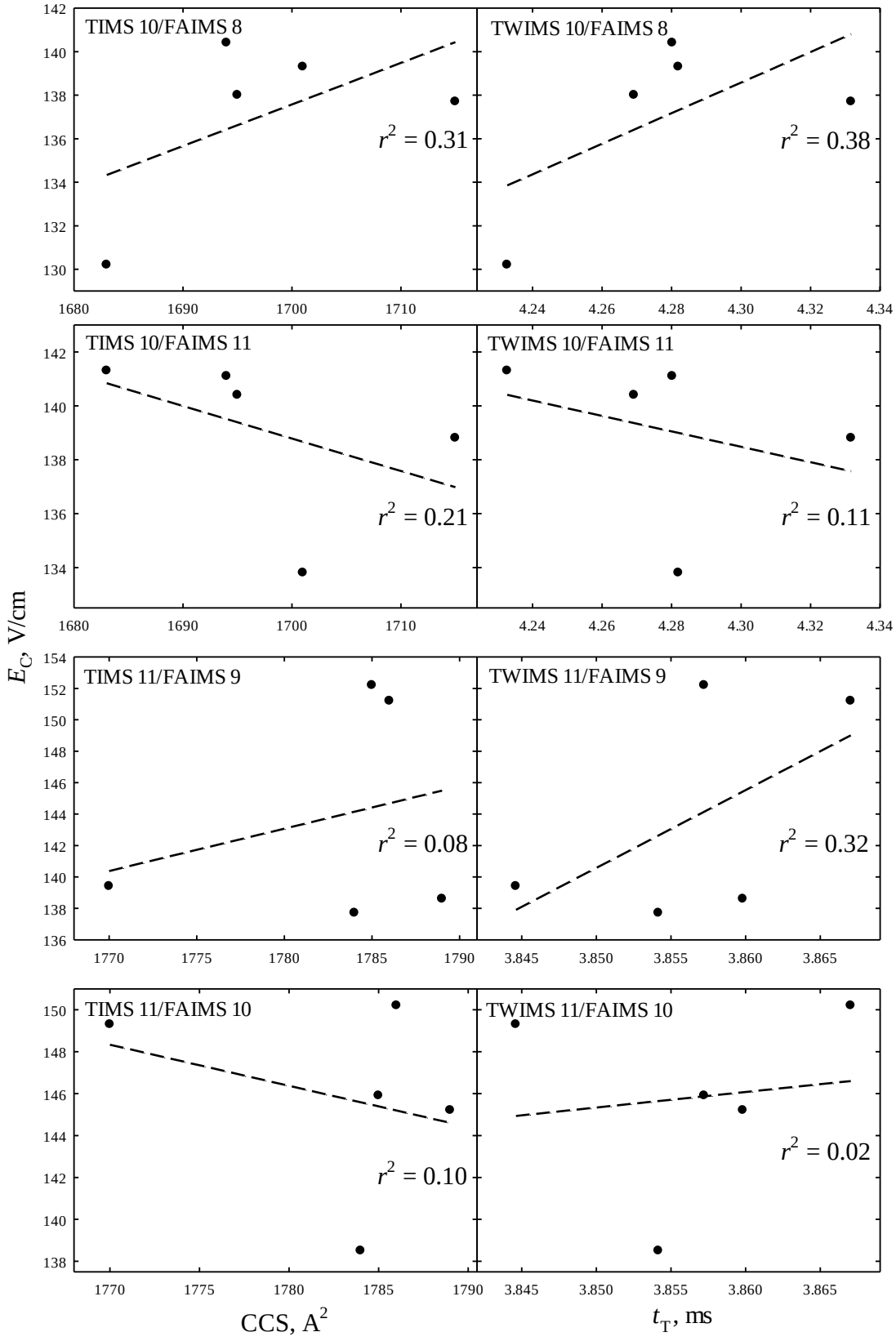


Figure S13b

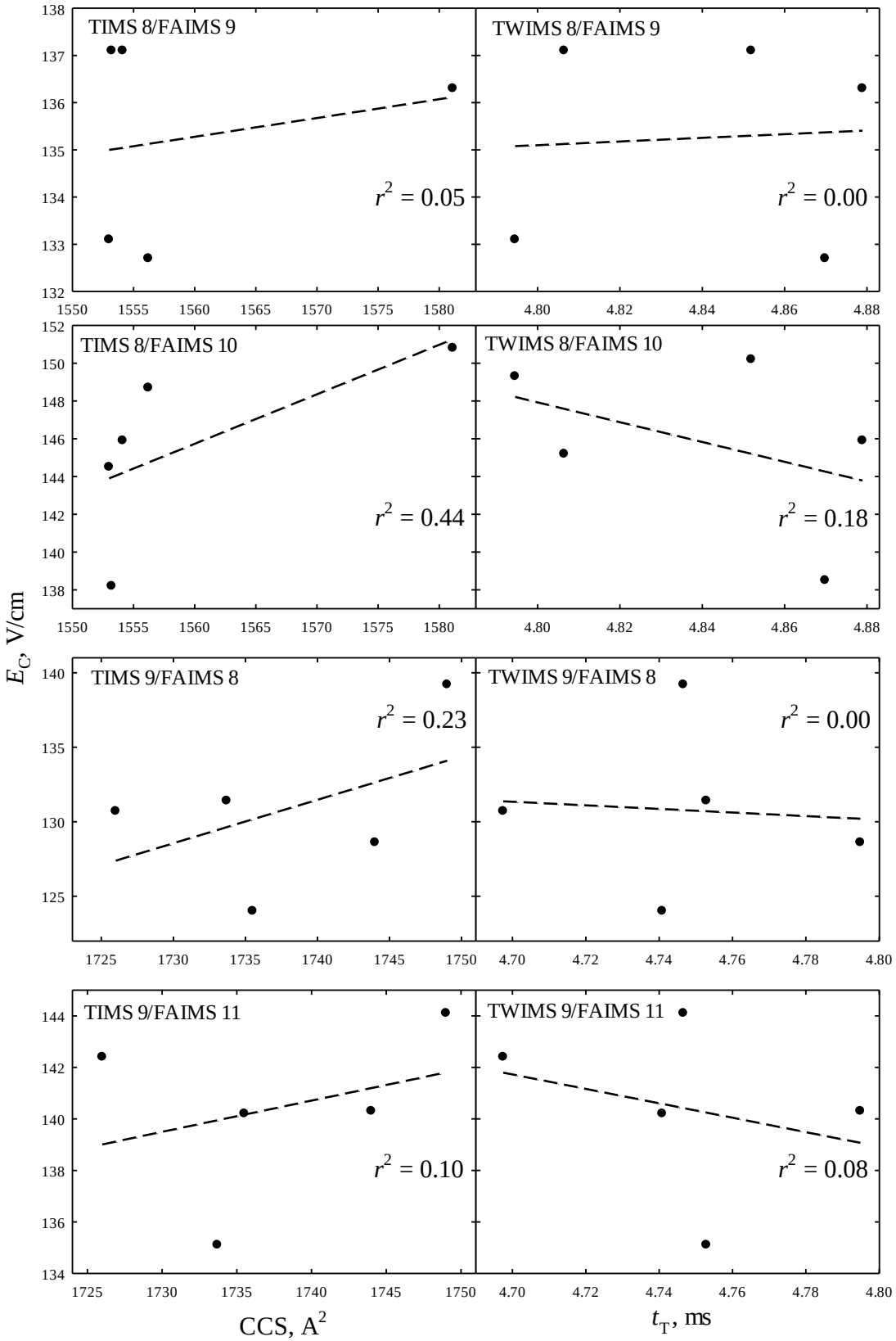

TIMS 8/FAIMS 9
TWIMS 8/FAIMS 9
r^2 = 0.05
r^2 = 0.00
TIMS 8/FAIMS 10
TWIMS 8/FAIMS 10
r^2 = 0.44
r^2 = 0.18
TIMS 9/FAIMS 8
TWIMS 9/FAIMS 8
r^2 = 0.23
r^2 = 0.00
TIMS 9/FAIMS 11
TWIMS 9/FAIMS 11
r^2 = 0.10
r^2 = 0.08
E_C, V/cm
CCS, A^2
t_T, ms

Figure S13b (continued)