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Evaluation of polystyrene-divinylbenzene cross-linked chromatographic media for separation of proteins

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Introduction

Reversed-phase chromatography of proteins coupled with mass spectrometry has become an important tool in the study of the proteome or characterization of pharmaceutical proteins. Silica based reversed-phase columns such as Symmetry300™C_4 and Delta-Pak™C_4 are widely used due to their excellent peak shape, peak capacity, and protein recovery. Since high and low pH mobile phases are frequently used in LC-MS methods for proteins, the life time of silica based columns may be compromised. Porous polystyrene-divinylbenzene (PS/DVB) is known to have excellent pH stability (1-14) for a broad range of mobile phases which facilitates column regeneration with strong base or acid. However, most commercially available PS/DVB media suffer from broad bands and low peak capacity. For developing better chromatographic media, a series of experimental porous poly-DVB media with various average pore sizes, surface areas, degree of cross-linking, and particle sizes were prepared. They were evaluated along with commercially available PS/DVB columns, silica based Symmetry300™C_4 and Delta-Pak™C_4 columns. Ribonuclease A, bovine serum albumin, ß-lactoglobulin A, and ovalbumin were used as a model sample to assess the PS/DVB media. Among them, bovine serum albumin, ß-lactoglobulin A, and ovalbumin are known as "bad" proteins for reversed-phase chromatography.[1] The column performance was evaluated by peak capacity and protein recovery using mobile phases containing TFA. Additionally, a mass spectrometry friendly solvent, mobile phases containing formic acid, was used to evaluate the columns for LC-MS conditions.

Experimental

Particle Synthesis and Characterization:

The porous polyDVB particles were prepared by suspension polymerization method. Elutriation was used to classify the particles. The size of particle was measured by Elzone Particle Size Analyzer. The synthesis variables such as reaction temperature, divinylbenzene and organic solvent contents were used to prepare a set of particles with distinct pore morphology. Particle surface morphology was examined by SEM and surface area was obtained by BET method. Average pore size of particles was examined by size-exclusion chromatography method. Degree of cross-linked was examined by ^{13}C CP/MAS NMR method.

Chromatographic Conditions:

HPLC System: Waters Alliance™ 2795 equipped with a 996 photodiode array detector.

LC/MS System: Waters CapLC®– Micromass LCT™

Columns: All columns were in 4.6 × 50 mm stainless steel configuration except for the columns used for Figure 2, 3 and 4 which are in 1.0 × 50 mm stainless steel configuration.

Protein Solution: Ribonuclease A (12.5µM), bovine serum albumin (33µM), ß-lactoglobulin A (25µM), and ovalbumin (6.65µM).

Injection volume: 20µL for 4.6 × 50 mm column, 4µL for 1.0 × 50 mm column.

Peak capacity: Calculated as $P = 1 + (t_g/w)$, where t_g = gradient time (15 minutes in this study) and w = average of peak width of four proteins at 50% peak height.

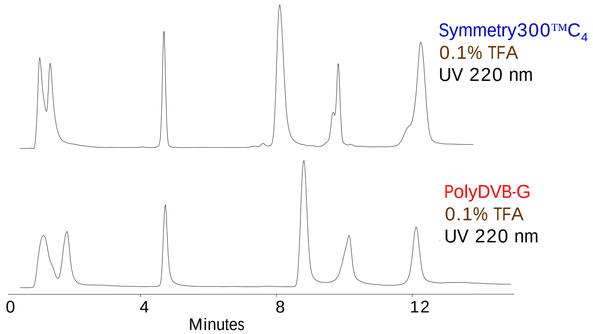

Figure 1. Comparison of protein separation on silica and polyDVB-G columns in 0.1% TFA. Mobile phase A: 0.1% TFA in water. B: 0.08% TFA in acetonitrile. Gradient from 20% to 65% B in15 min., 0.75ml/min, 40°C, UV 220nm, 4.6 × 50 mm columns.

Table 1. Material properties and chromatographic performance in 0.1% TFA

Material ID	Pore size (Å)	Surface area (m^2/g)	Cross-linked (%)	Particle size (µm)	Peak Capacity
PolyDVB-A	420	505	28	3.5	140
PolyDVB-B	450	383	17	3.8	136
PolyDVB-C	140	549	22	3.9	117
PolyDVB-D	200	511	24	4.1	124
PolyDVB-E	210	397	23	4.7	138
PolyDVB-F	290	504	21	4.8	137
PolyDVB-G	140	709	33	5.7	104
PolyDVB-H	450	383	17	5.8	120
PolyDVB-I	140	549	22	5.8	120
PolyDVB-J	190	519	28	4.1	122
PolyDVB-K	77	683	26	5.5	83
PolyDVB-L	89	489	15	4.7	85
PolyDVB-M	180	546	14	3.4	136
PolyDVB-N	180	546	14	4.9	123
PolyDVB-O	180	546	14	6.4	107
PolyDVB-P	190	519	28	7.0	101
Symmetry 300™ C_4	277	116	____	3.5	139

Note: Protein recovery of Symmetry300™ C_4 is ~100% for Ribonuclease A, ~91% for bovine serum albumin, ~38% for ß-lactoglobulin A, and ~46% for ovalbumin. Protein recovery of PolyDVB columns is varied between ~ 95 to 100% for Ribonuclease A, ~ 90 to 100% for bovine serum albumin, ~35 to 53% for ß-lactoglobulin A, and ~45 to 60% for ovalbumin, except PolyDVB-D.

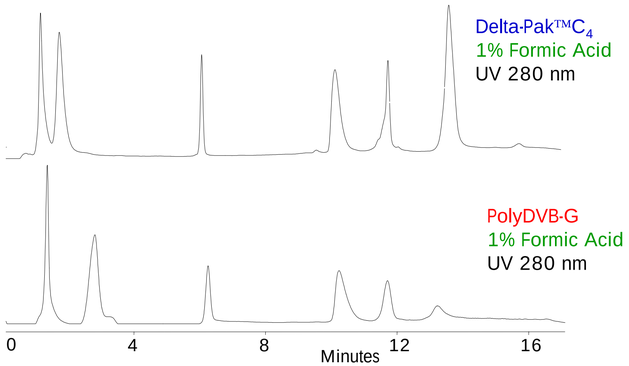

Figure 2. Comparison of protein separation on silica and polyDVB-G columns in 1% formic acid. Mobile phase A: 1% formic acid in water. B: 0.65% formic acid in acetonitrile. Gradient from 10% to 55% B in15 min., 35.4µL/min, 40°C, UV 280nm, 1.0 × 50 mm columns.

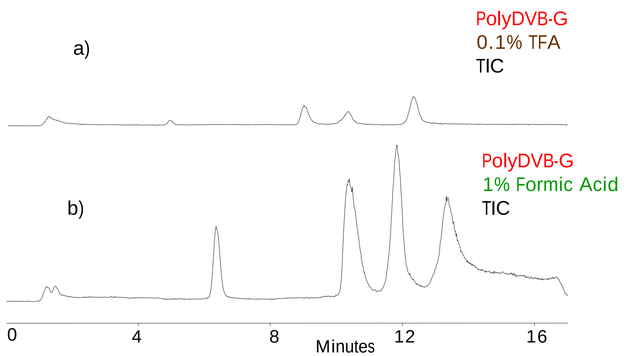

Figure 3. Comparison of LC/MS total ion chromatograms of protein separation used 0.1% TFA or 1% Formic acid. a) Mobile phase A: 0.1% TFA in water. B: 0.08% TFA in acetonitrile, Gradient from 20% to 65% B in15 min., b) Mobile phase A: 1% formic acid in water. B: 0.65% formic acid in acetonitrile, Gradient from 10% to 55% B in15 min., 35.4µL/min, 40°C, 1.0 × 50 mm columns. TOF MS conditions: Source = ESI (+), Capillary (kV) = 2.5, sample cone (V) = 35, desolvation temp. 250°C, source temp. 100°C.

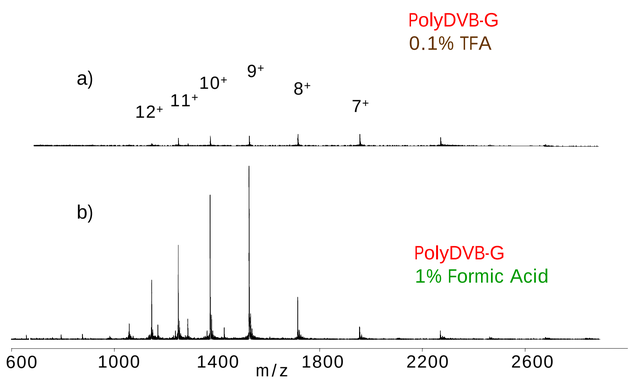

Figure 4. Comparison of Ribonuclease A mass charge envelope from the combined ion spectrum over the peak eluted with 0.1% TFA or 1% Formic acid. a) Mobile phase A: 0.1% TFA in water. B: 0.08% TFA in acetonitrile., Gradient from 20% to 65% B in15 min., b) Mobile phase A: 1% formic acid in water. B: 0.65% formic acid in acetonitrile, Gradient from 10% to 55% B in15 min., 35.4µL/min, 40°C, 1.0 × 50 mm columns. TOF MS conditions: Source = ESI (+), Capillary (kV) = 2.5, sample cone (V) = 35, desolvation temp. 250°C, source temp. 100°C.

Conclusion

- The chromatographic performance of polyDVB media is highly dependent on the synthesis formulas.
- Poly-DVB media can be made to perform as well as the best silica based columns in terms of peak capacity and protein recovery.
- PolyDVB media is excellent for using formic acid.
- PolyDVB media have the potential to be very useful columns for LC-MS of protein analysis applications, though more research and development is needed.
- Mobile phases containing formic acid give better mass signal than mobile phases containing TFA.

References

1. Burton, W. G., Nugent, K. D., Slattery, T. K., and Summers, B. R. *J. Chromatogr.*, 433, 363-379, (1988).