Superficially porous particles with carbon core and nanodiamond–polymer shell for protein separations

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Current trends in column technology

Core-shell small particles (1.3-1.9 µm)

Fully porous small particles (1.5-1.9 µm)

Silica monolith (2nd generation)

Organic polymer monoliths

\[ N_{\text{max}} \sim 500'000 \text{ plates/meter} \]

\[ N_{\text{max}} \sim 300'000 \text{ plates/meter} \]

\[ N_{\text{max}} \sim 170'000 \text{ plates/meter} \]

\[ N_{\text{max}} \sim 140'000 \text{ plates/meter} \]

Close to 1'000 columns are commercially available for HPLC!

very fast
very efficient
very high pressure

Parallel segmented flow
PLOT columns
Silica colloidal crystals
Pillar arrays
Ordered structures
New carbon-diamond based superficially porous material

**FLARE columns**

- Unique selectivity relative to silica
- pH stability: $1 < \text{pH} < 13$
- Thermally stable up to $100 \, ^\circ\text{C}$

[Graph showing chromatograms for different pH values with peaks labeled for CBD, CBN, Δ9-THC, and Δ8-THC]
Other possible applications?

For large biomolecules?!

1) It possesses an average pore size of 250 Å

2) No residual silanols

3) Retention mechanism and selectivity are different compared to silica based materials (hydrophobic + anion-exchange)

4) It is stable at high temperature (recovery, effective molecular diffusion)

5) Diamond and carbon have advantageous heat properties (important for large solutes’ retention control) – less frictional heating – no negative impact on retention and band broadening

6) It offers the benefits of core–shell (pellicular) particle technology
Column efficiency, plate height equations

\[ h = f(\nu) \]

The old empirical formula (1956):

\[ h = a + \frac{b}{\nu} + c \nu \]

„a“ – eddy dispersion

„b“ - longitudinal diffusion

„c“ mass transfer resistance

More sophisticated recent models (Knox, Golay, Giddings, Horvath, Huber, Myabe, Guiochon...):

\[ h = a \nu^{1/3} + \frac{b}{\nu} + c_f \nu + c_p \nu + h_{heat} \]

„c_f“ - film or „external"

„c_p“ - transparticle or „internal“

\[ h = a \nu^{1/3} + \frac{b}{\nu} + c_f \nu + c_p \nu + \frac{1}{(1/ 2 \lambda_{heat}) + (1/ 2 \omega_{heat} \nu)} \]

eddy dispersion coefficient related to a flow exchange mechanism generated by heat friction in the column

eddy dispersion coefficient related to a diffusion exchange mechanism (Aris) generated by heat friction in the column
Why superficially porous particles?

**Core-shell advantages:**

1) More advantageous $C_p$ term (for large molecules) (Horváth in the late 60’s)
2) lower $B$ term (for small molecules)
3) lower $A$ term...

**Compromises:**
- Decrease in retention
- Lower loading capacity

**Optimal structure:**
Compromise, $0.6 < \rho < 0.9$
(porous volume fraction between 80 and 20 %)
The impact of particle structure

volume fraction

The figure shows the relationship between the volume fraction of porous material and the solid core to core-shell ratio ($\rho$). The graph indicates that the volume fraction decreases as the solid core to core-shell ratio increases. The specific values for different materials are shown, such as Halo, Ascentis Express, Poroshell, BrownLee, Kinetex 1.3, Kinetex 2.6, Kinetex 5, Halo 5, Aeris WP, and Poroshell 300.

expected gain in efficiency

The graph on the right illustrates the expected gain in efficiency with respect to the relative plate height contribution (relative scale). The curves represent different materials' performance, with relative $h_{\text{C, particle}}$ and relative $h_{\text{C, film}}$.

loadibility

The loadibility of the materials is indicated by the graph's position on the y-axis, which represents the volume fraction of porous material.

phase ratio

The phase ratio is shown by the graph's position on the x-axis, which represents the solid core to core-shell ratio ($\rho$).

retention

The retention of the materials is indicated by the graph's position on the graph spaces.

3.6 µm, 1 µm

The figure shows the size difference between two particle structures, with 3.6 µm and 1 µm being indicated.

$\rho = 0.94$

The figure indicates a volume fraction of approximately 16%, with $\rho = 0.94$.

Theoretical $h - \nu$ plots

Theoretical $h-\nu$ curves of fully porous and core-shell packing ($\rho=0$, $\rho=0.63$, $\rho=0.87$ and $\rho=0.73$). Hypothetical mobile phase composition: 50 % acetonitrile – 50 % water, and analyte molecular weight: 1000 g/mol. No extra-column band broadening is assumed.
The effect of molecular diffusion on plate height

For proteins, the C-term is the most important contribution.

B-term region

10 kDa protein

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Gradient elution, peak capacity

Peak capacity describes the column performance in gradient elution mode

$$P = 1 + \frac{t_g}{1.7 \cdot w_{50\%}}$$

Corresponds to a resolution of 1 between consecutive peaks

In analogy with isocratic plate height ($H$) equations, when combining isocratic plate height and gradient peak capacity equations the next formula can be written:

$$P = \frac{1}{4} \sqrt{A - (2\gamma D_M t_0) / L} \sqrt{C \left( \frac{1}{D_S} \right) \left( \frac{L}{t_0} \right)} + \frac{1}{G + 1} \ln \left[ \frac{k_{0,\text{max}}}{k_{0,\text{min}}} \right]$$

the effect of molecular diffusivity and flow rate (that can be expressed with $t_0$) on the peak capacity can directly be highlighted.
Peak capacity with model proteins on FLARE

100 x 2.1 mm FLARE column, mobile phase A: 0.2% TFA, mobile phase B: 0.2% TFA in ACN, gradient: 20 – 45 %B

**Effect of temperature**

- 50°C
- 30°C

**Effect of flow-rate**

- 0.4 ml/min
- 0.2 ml/min

Impact of TFA%

- Increase in retention
- Improvement in peak capacity
- Decrease in overall polarity (increase in hydrophobicity) of the proteins as the TFA concentration increased
- Decrease in polarity of the stationary phase through ion pairing effects of TFA

0.2 – 0.5% TFA is required

Temperature, additive, flow-rate

Differences compared to silica-based „conventional” columns

No need to work at high temperature (avoiding on-column protein degradation)

Fast gradients can be applied (without loosing peak capacity)

Higher amount of TFA is required (0.2 - 0.5%)

Useful for fast gradient protein separations performed at low temperature!
Real life protein separations

Why using RP-HPLC?
- Assay (protein content)
- Protein impurities
- Degradants of proteins (oxidation, reduction, deamidation)
- Small Aggregates (dimer)
- Heterogeneity (charge, isoforms)
- Heavy and light chain variants (mAb)
- Fab and Fc variants, fragments (mAb)
- Investigation of disulphide bridges
- Excipients (polysorbate-20, -80)
- Amino Acid Analysis (AAA)
- Peptide maps

Closely related proteins have to be separated!

Method development

\[ R = \left( \frac{\sqrt{N}}{4} \right) \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k}{k + 1} \right) \]

- Very efficient recent columns
- Optimization of column length
- Increasing temperature

- Organic modifier
- pH
- Temperature
Separation of interferon variants

Column: FLARE 100 x 2.1 mm C18 M, Mobile phase „A”: 0.3 % TFA, „B”: 0.3% TFA in ACN, gradient: 25-50 %B in 7 min, flow rate: 0.3 mL/min, temperature: 30 °C. Detection: fluorescence (λex: 280 nm, λem: 360 nm, 20 Hz sampling rate).

Separation of filgrastim variants

Column: FLARE 100 x 2.1 mm 16636.1-2, Mobile phase „A”: 0.5 % TFA, „B”: 0.5% TFA in ACN, gradient: 37-52 %B in 4 min, flow rate: 0.2 mL/min, temperature: 60 °C. Detection: UV (280 nm, 20 Hz sampling rate).
Filgrastim pharmacopeia method (related proteins)

Column:
- *size*: \( l = 0.15 \text{ m}, \bar{d} = 4.6 \text{ mm} \);
- *stationary phase*: octadecylsilyl silica gel for chromatography R (3 \( \mu \text{m} \)) with a pore size of 20 nm;
- *temperature*: 65 °C.

Mobile phase:
- *mobile phase A*: mix 499 ml of acetonitrile for chromatography R and 500 ml of water R and add 1 ml of trifluoroacetic acid R;
- *mobile phase B*: mix 49 ml of water R and 950 ml of acetonitrile for chromatography R and add 1 ml of trifluoroacetic acid R;

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase A (per cent V/V)</th>
<th>Mobile phase B (per cent V/V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 4</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>4 - 19</td>
<td>92 → 72</td>
<td>8 → 28</td>
</tr>
<tr>
<td>19 - 19.1</td>
<td>72 → 0</td>
<td>28 → 100</td>
</tr>
<tr>
<td>19.1 - 21</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>21 - 21.1</td>
<td>0 → 92</td>
<td>100 → 8</td>
</tr>
<tr>
<td>21.1 - 25</td>
<td>92</td>
<td>8</td>
</tr>
</tbody>
</table>

*Flow rate*: 1.0 ml/min.

United States Pharmacopeia (USP)
Peak capacity (filgrastim)

Impact of gradient time (steepness) at 60 °C

Impact of temperature at 6.25 %B/min gradient steepness (4 min long gradient)

Mobile phase „A”: 0.5 % TFA, „B”: 0.5% TFA in ACN, gradient: 35-60 %B (on FLARE) and 50-75 %B (on AERIS), flow rate: 0.2 mL/min. Columns: 100 x 2.1 mm
mAbs represent an important class of therapeutic proteins with a wide range of clinical indications.

Chemical and enzymatic modifications during manufacture, formulation, storage.

Intrinsic microheterogeneity

Common modifications:
  N-glycosylation
  Methionine oxidation
  Fragmentation
  Lysine truncation
  Deamidation
  Fab amino termini variants (glutamine – pyroglutamate)

Their detailed characterization requires several orthogonal methods/tools.

Among them RPLC is an important tool.
Strategies, approaches in LC for mAb analysis

1. Complete proteolytic digestion of a mAb (peptide mapping, „bottom-up” approach)
2. Analysis of fragments/domains: HC, LC, Fab, Fc, F(ab')2, sFc, Fd („middle down”)
3. Analysis of intact mAbs („top down”)

Reversed phase (RP-HPLC):
• intact mAb (heterogeneity, similarity)
• HC-LC (oxidation, variability)
• Fab-Fc, sFc-Fd (variability, post-translational modifications, such as N-terminal cyclization, oxidation, deamidation, and C-terminal processed lysine residues)
• Peptide mapping (primary structure)

Possible issues
- Large hydrodynamic radii (≥ 35Å)
- Huge number of charges (≥ 100)
- Low diffusion coefficient

**Adsorption**
**Peak broadening**
**Peak tailing**
Recovery of intact mAb (rituximab IgG1)

Column: BEH300 C4 (150 mm x 2.1 mm), temperature: from 40 °C up to 80 °C, injected volume: 0.5 µL, detection: 280 nm. Mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile. Gradient: from 30% to 37% B in 6 min, flow rate: 0.3 mL/min.

Variants of the intact mAb can be separated.

Intact and reduced mAb (bevacizumab IgG1)

Column: FLARE 100 x 2.1 mm C18 M, Mobile phase „A”: 0.2 % TFA, „B”: 0.2% TFA in ACN, gradient: 20-35 %B in 4 min, flow rate: 0.3 mL/min, temperature: 90 °C. Detection: UV (280 nm, 20 Hz sampling rate).
Heavy chain variability (IgG2 panitumumab)

Column: FLARE 100 x 2.1 mm C18, Mobile phase “A”: 0.5 % TFA, “B”: 0.5% TFA in ACN, gradient: 25-33 %B in 10 min, flow rate: 0.15 mL/min, temperature: 90 °C. Detection: FL (280-360 nm).
Trastuzumab (IgG1) papain digested sample

Column: FLARE 100 x 2.1 mm 166636.1-2, Mobile phase „A”: 0.5 % TFA, „B”: 0.5% TFA in ACN, gradient: 20-35 %B in 4 min, flow rate: 0.2 mL/min, temperature: 90 °C. Detection: UV (280 nm, 20 Hz sampling rate).
Papain digested + reduced IgG1 (rituximab)

Column: FLARE 100 x 2.1 mm C18, Mobile phase „A”: 0.5 % TFA, „B”: 0.5% TFA in ACN, gradient: 25-33 %B in 10 min, flow rate: 0.15 mL/min, temperature: 90 °C. Detection: FL (280-360 nm).
Brentuximab-Vedotin was digested with endoproteinase Lys-C

Column: FLARE 100 x 2.1 mm 16636.1-2, Mobile phase „A”: 0.5 % TFA, „B”: 0.5% TFA in ACN, gradient: 0-50 %B in 12 min, flow rate: 0.2 mL/min, temperature: 60 °C. Detection: UV (280 nm, 20 Hz sampling rate).
Conclusion

• This carbon-based nanodiamond-polymer shell material is interesting for the separation of large molecules

• With most proteins, there is no need to work at high temperature

• When high temperature is required (e.g. mAbs), it is again useful since it can be operated at up to 100 °C

• The achievable peak capacity is comparable to the best silica-based wide-pore materials

• The retention is somewhat lower compared to silica-based fully porous and core-shell materials but can easily be adjusted by decreasing the solvent-strength or by the higher amount of TFA
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Thank you for your attention!