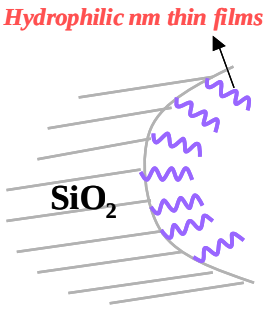
# Sepax Nanofilm SEC Phases

## General Description

Utilizing proprietary surface technologies, Sepax *Nanofilm* SEC phases are made of the uniform, hydrophilic, neutral, and polymeric thin films chemically bonded on the high purity and enhanced mechanical stability silica. Sepax *Nanofilm* SEC phases have been innovatively and specially designed to ensure highest resolution and maximum recovery for the separation of biological molecules, such as proteins, nucleic acids, peptides and others. These polymer coatings are a few of nanometer thick. The Sepax proprietary surface technologies allow the chemistry of thin film formation completely controlled that results in high column-to-column reproducibility. The nature of the chemical bonding and densely packed polymer thin film benefit *Nanofilm* SEC phases with exceptional stability. The uniform polymer coating enables high efficiency separation. The narrow dispersed, spherical silica particles of the *Nanofilm* packings for SEC-150, SEC-250, SEC-500, and SEC-1000 have nominal pore sizes at 150 Å, 250 Å, 500 Å, and 1,000 Å, respectively. Their pore volume is *ca.* 0.9 mL/g. *Nanofilm* SEC columns are packed with a proprietary slurry technique to achieve uniform and stable packing bed density for maximum column efficiency. Typical applications for *Nanofilm* SEC columns are the separations of biological molecules in aqueous buffer solutions.

## Surface Structure




## Characteristics

- *High efficiency and resolution*
- *Extremely stable in high salt solution*
- *Great reproducibility*
- *Protein separation over very low salt concentration*
- *High protein recovery and maintaining biological activity*
- *Wide pH application range 2 - 9*

## High Efficiency Separation

For biological separations, nonspecific interactions with the packing materials are the major contribution for low separation efficiency, such as peak tailing, as well as low recovery. The unique surface chemistry of *Nanofilm* SEC packings generates uniform polymer chains covalently bonded on the silica. Such a uniform stationary phase is neutral and hydrophilic, which has negligible nonspecific interactions with biological molecules, especially proteins. This special coating allows *Nanofilm* SEC columns to achieve high selectivity and high efficiency separations. For example, uracil, as a test compound could reach as high efficiency as 90,000 plates per meter, as shown in Table 1.

Table 1. Column efficiencies (measured using uracil in 0.1 M phosphate buffer, pH 7.0)

| | |
|---|---|
| Sepax*Nanofilm* SEC-150 | >90,000 plates/meter |
| Sepax*Nanofilm* SEC-250 | >90,000 plates/meter |
| Sepax*Nanofilm* SEC-500 | >85,000 plates/meter |
| Sepax*Nanofilm* SEC-1000 | >85,000 plates/meter |

### *Separation of Sepax Standard Proteins*

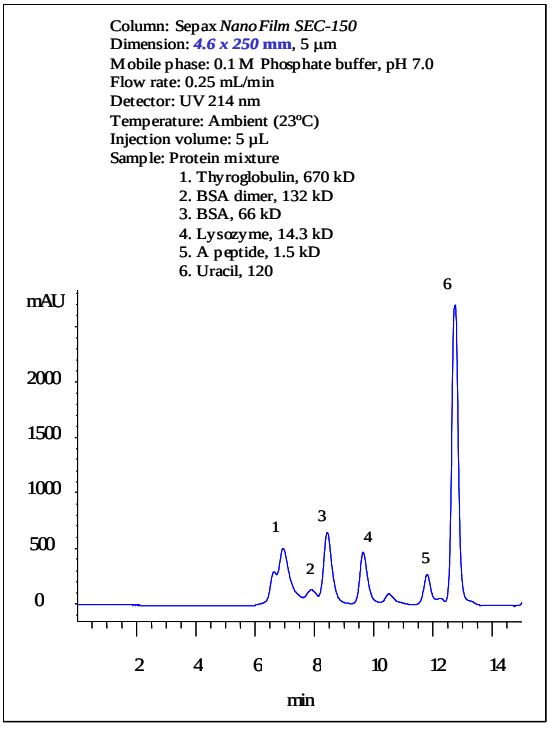



Figure 1. Separation of 4 proteins and a peptide by using a 4.6x250 mm *Nanofilm* SEC-150 column.

The first example for high efficiency separation is shown in Figure 1. Four proteins (thyroglobulin, BSA dimer, BSA, lysozyme) and a peptide are well separated with 30,000 plates per meter for lysozyme. Even an impurity from lysozyme was very well separated. This was only seen separated with high efficiency capillary electrophoresis. (Reference: Sepax Capillary Electrophoresis Catalog). For standard test of size exclusion columns, manufacturers normally use Biorad standard proteins (thyroglobulin, IgG, ovalbumin, and myoglobin). The reason is that those proteins have pI less than 7.5, which are not positively charged at pH 7.0 separation condition. The highly positively charged proteins, such as lysozyme (pI=11.0), cytochrome C (pI= 10.6), aprotin (pI=11.0) are very difficult to elute due to the strong electrostatic interactions with the surface of the packing materials manufactured by other vendors. However, this is no longer a problem for Sepax *Nanofilm* SEC columns. Sepax sets its own standard of test proteins including lysozyme, a very sensitive protein for testing the quality of bioseparations.

### *Separation of BSA dimer from BSA protein*

The second example for high efficiency *Nanofilm* SEC columns is the separation of BSA dimer from BSA, as shown in Figure 2. A *Nanofilm* SEC-250 column, 4.6x300 mm, achieved baseline separation of BSA dimer from BSA at 150 mM phosphate, pH 7.0.

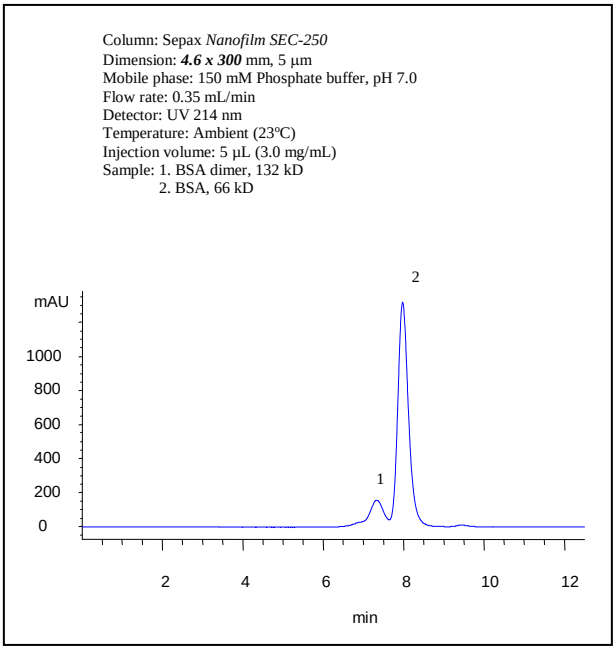



Figure 2. Separation of BSA dimer from BSA by using a 4.6x300 mm *Nanofilm* SEC-250 column.

### *Separation of the aggregates from thyroglobulin*

Thyroglobulin is a huge protein (MW 670,000). The commercial sample of thyroglobulin contains the aggregate impurity, possibly dimer or tetramer. Sepax *Nanofilm* SEC-500 provides a baseline separation of the aggregates from thyroglobulin, as shown in Figure 3.

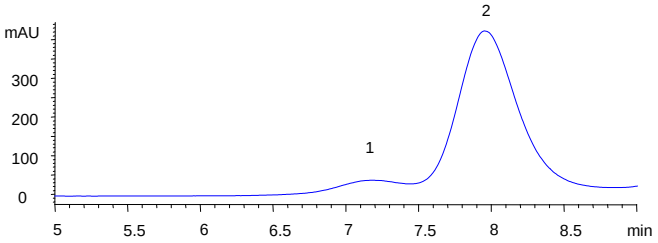



Figure 3. Separation of the aggregates from thyroglobulin by a *Nanofilm* SEC-500 column (5 µm, 4.6x300 mm). The separation conditions: mobile phase, 150 mM Phosphate buffer, pH 7.0; flow rate, 0.35 mL/min; detection: UV 214 nm; temperature, ambient (23 ºC); and injection volume, 5 µL. The protein sample: (1) the aggregates; and (2) Thyroglobulin (1.0 mg/mL), 670 kD.

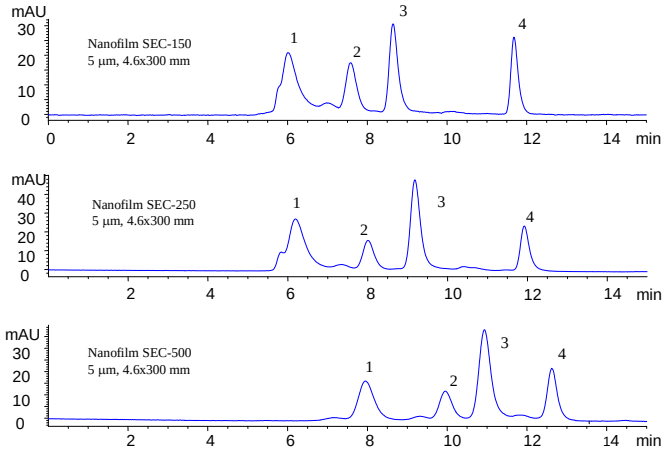



Figure 4. Comparison of the separation patterns by *Nanofilm* SEC-150, *Nanofilm* SEC-250 and *Nanofilm* SEC-500. The separation conditions: mobile phase, 150 mM Phosphate buffer, pH 7.0; flow rate, 0.25 mL/min; detection: UV 280 nm; temperature, ambient (23 oC); and injection volume, 5 µL. The protein mixture: (1) Thyroglobulin (1.0 mg/mL), 670 kD; (2) BSA (1.0 mg/mL), 66 kD; (3) Ribonuclease A (1.0 mg/mL), 13.7 kD, and (4) Uracil (2.5 µg/mL), 120

### *The Impact of Pore Structure on Separation*

The pore size of the SEC packings plays a critical role on protein separations. The selection of a *Nanofilm* SEC phase for a particular molecular weight range protein sample is based on the exclusion limit and linear molecular weight range of the *Nanofilm* packings that are shown below.

| *Nanofilm* Packings | Protein MW exclusion limit |
|---|---|
| SEC-150 | 750,000 |
| SEC-250 | 1,250,000 |
| SEC-500 | 2,500,000 |
| SEC-1000 | 5,000,000 |

To demonstrate the impact of the *Nanofilm* SEC packings with different pore size on the separation pattern, Figure 4 shows the separation of three proteins and a small molecule with the MW range from 120 to 670,000.

## High Protein Recovery and Maintaining Activity

The *Nanofilm* SEC phases are composed of a hydrophilic and neutral polymer thin film with certain thickness that is chemically bonded on the silica surface. Proteins and other biological molecules have negligible nonspecific interactions with such stationary phases. The protein adsorption to the silica is suppressed, leading to high recovery and maintaining the activity after separations. Table 2 shows the recovery results of BSA and lysozyme, the representatives for acidic and basic proteins, separately.

Table 2. Recovery of proteins from the *Nanofilm* SEC columns (measured with a 4.6x250 mm column in 0.15 M phosphate buffer, pH 7.0)

| Column | Protein | Recovery % |
|---|---|---|
| *Nanofilm* SEC-150 | BSA | >95% |
| | Lysozyme | >95% |
| *Nanofilm* SEC-250 | BSA | >95% |
| | Lysozyme | >95% |
| *Nanofilm* SEC-500 | BSA | >95% |
| | Lysozyme | >95% |
| *Nanofilm* SEC-1000 | BSA | >95% |
| | Lysozyme | >95% |

## High Stability of Sepax Nanofilm SEC Phases

The *Nanofilm* SEC columns uses full coverage bonded silica packing. The polymeric thin film is densely packed on the silica surface which greatly hinders the diffusion of the attacking molecules to the silica-polymer bonding, thus enabling exceptional high stability. The *Nanofilm* SEC phases are compatible with most aqueous buffers, such as ammonium acetate, phosphate, trizma and so on. When 150 mM phosphate buffer at pH 7.0 was used as the mobile phase to run the *Nanofilm* SEC columns, 1,000 injections or 3 months of usage made negligible deterioration for its performance. After 3 months of running in 150 mM phosphate buffer at pH 7.0, the average retention time change was within 5%.

Figure 5 shows the separation profiles of a *Nanofilm* SEC-150 at the day 1, 5, and 10 with 10 hours/day running at the flow rate of 0.35 mL/min in 150 mM and 100 mM phosphate buffer, respectively. The separations are almost identical.

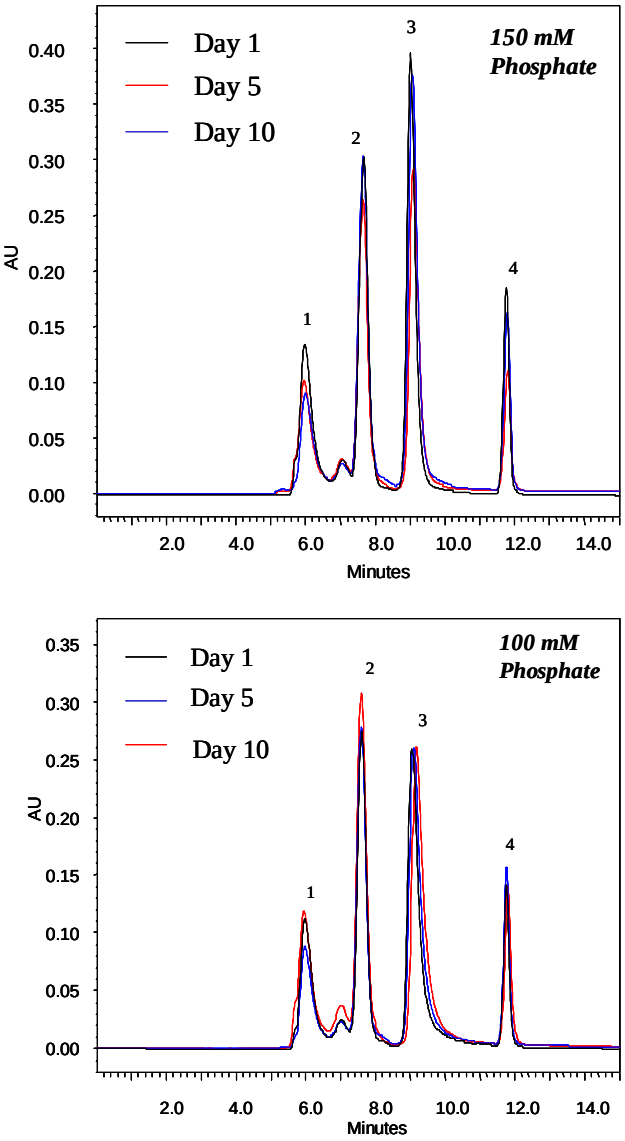



Figure 5. Column: *Nanofilm* SEC-150 (5mm, 4.6x300mm)
Mobile phase: 150 mM and 100 mM PBS, pH 7.0
Flow rate: 0.35 mL/min
Running for 10 h/day
Temperature: Ambient
Sample: 1. Thyroglobulin (1.0 mg/mL)
2. BSA (1.0 mg/mL)
3. Lysozyme (1.0 mg/mL)
4. Uracil (50 μg/mL)

Figure 6 is the stability test for retention time of proteins and a small molecule over 500 column volume running in two weeks. It shows negligible retention time change for each of those three proteins as well as uracil within 500 column volume testing.

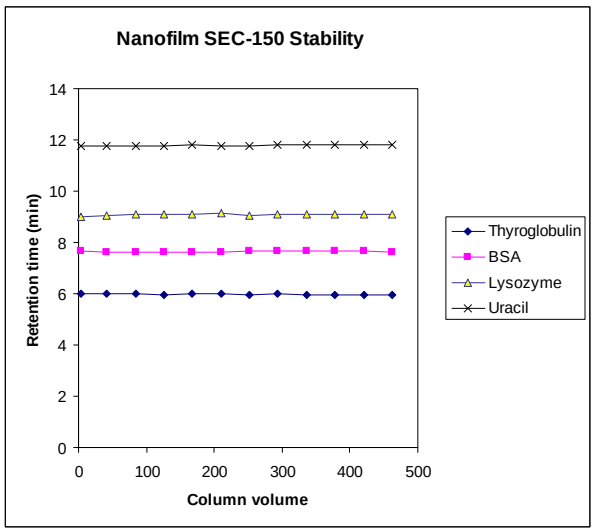



Figure 6. Column: *Nanofilm* SEC-150 (5mm, 4.6x300mm)
Mobile phase: 150 mM and 100 mM PBS, pH 7.0
Flow rate: 0.35 mL/min
Running for 10 h/day
Temperature: Ambient
Sample: 1. Thyroglobulin (1.0 mg/mL)
2. BSA (1.0 mg/mL)
3. Lysozyme (1.0 mg/mL)
4. Uracil (50 μg/mL)

The *Nanofilm* SEC phases can tolerate very high concentration of salts, such as 1.0 M. The *Nanofilm* SEC columns are very stable in both organic solvents, such as methanol, ethanol, THF, DMF, DMSO, and so on, and the mixture of water and organic solvents.

The *Nanofilm* SEC columns have very good stability over a wide range of pH from 2 to 8.5. In addition, they can even tolerate very higher pH, such as pH 8.5-10 for temporary using.

The tolerance of temperature for the *Nanofilm* SEC phases is also excellent. The working temperature can go as high as 80 °C.

## *Reproducibility of the* Nanofilm *SEC columns*

The controlled surface chemistry for synthesizing the *Nanofilm* SEC phases makes the surface coating very reproducible, leading to very consistent columns. The separation variation from batch to batch is within 5% for the retention time. Figure 7 is the separation of the Sepax standard protein mixture by the *Nanofilm* SEC-250 columns from two different lots. The largest variation is the retention time for lysozyme, which is *ca.* 2%.

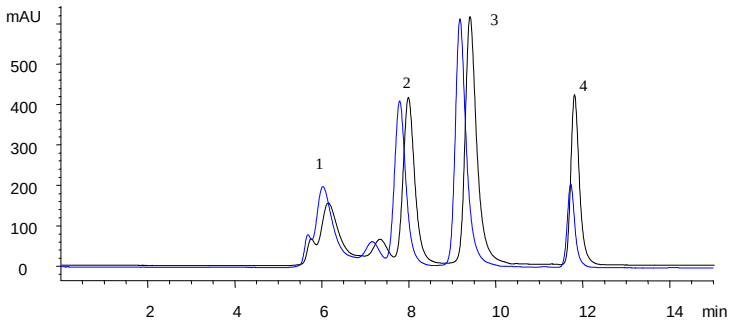



Figure 7. Comparison of the separations by two different batches of *Nanofilm* SEC-250 (5 μm), 4.6x300 mm columns. The separation conditions: mobile phase, 150 mM Phosphate buffer, pH 7.0; flow rate, 0.35 mL/min; detection: UV 214 nm; temperature, ambient (23 °C); and injection volume, 5 μL. The protein mixture: (1) Thyroglobulin (1.0 mg/mL), (2) BSA (1.0 mg/mL), (3) Lysozyme (1.0 mg/mL), and (4) Uracil (2.5 μg/mL).

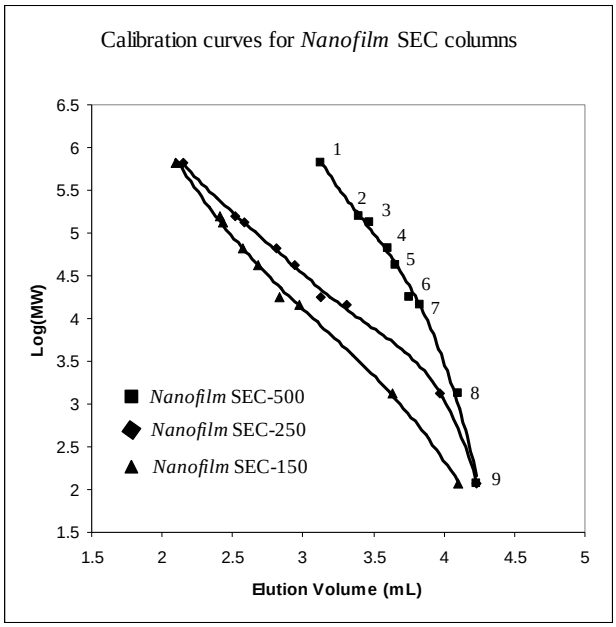



Figure 8. Protein MW calibration with *Nanofilm* SEC-150, *Nanofilm* SEC –250, and *Nanofilm* SEC–500 phases.
Columns: 4.6x300 mm, 5 μm
Mobile phase: 150 mM Phosphate buffer, pH 7.0
Flow rate: 0.25 mL/min
Detector: UV 214 nm
Injection volume: 5 μL
Sample: 1. Thyroglobulin, 670 kD
2. IgG, 150 kD
3. BSA dimer, 132 kD
4. BSA, 66 kD
5. Ovalbumin, 44 kD
6. Myoglobin, 17.6 kD
7. Lysozyme, 14.3 kD
8. B12, 1.35 kD
9. Uracil, 120

## MW Calibration for Protein Separation

For size exclusion chromatography, individual pore size packings determine the range of molecular weight for separation, while the pore volume controls the separation capacity. Sepax's four pore size packings cover whole range of biological molecules. The protein calibration curves for *Nanofilm* SEC-150, *Nanofilm* SEC-250, and *Nanofilm* SEC-500 are shown in Figure 8.

Another widely used method to characterize the SEC phases is the calibration curve between protein MW and the distribution coefficient ($K_d$). $K_d$ value is defined by the equation below:

$$K_d = (V_e-V_o)/(V_T-V_o)$$

Where $V_e$, $V_T$, and $V_o$ are the sample elution volume, the column total liquid volume, and the column void volume, respectively. The linear region between the protein MW and $K_d$ is frequently used as the reference for the selection of the SEC phases. As plotted in Figure 9, the *Nanofilm* SEC-150 phase has an excellent linear relationship between protein MW and $K_d$ in a wide range of MW.

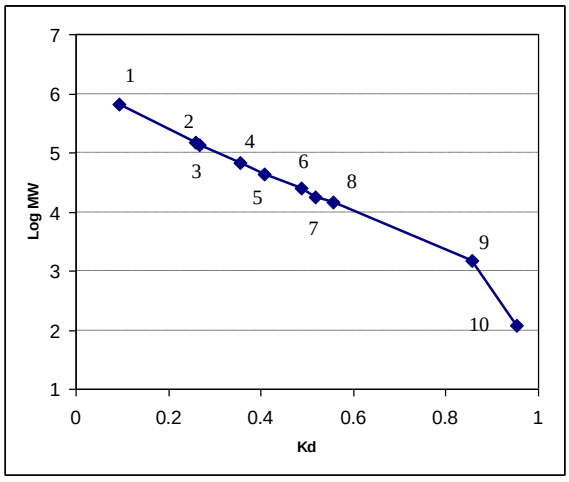



Figure 9. Protein MW calibration curve for *Nanofilm* SEC-150
Column: *Nanofilm* SEC-150, 4.6x150 mm, 5 µm
Mobile phase: 100 mM Phosphate buffer, pH 7.0
Flow rate: 0.25 mL/min
Detector: UV 214 nm
Temperature: Ambient (23 °C)
Injection volume: 5 µL
Sample: 1. Thyroglobulin, 670 kD
2. IgG, 150 kD
3. BSA dimer, 132 kD
4. BSA, 66 kD
5. Ovalbumin, 44 kD
6. Alpha-chymotrypsin, 25 kD
7. Myoglobin, 17.6 kD
8. Lysozyme, 14.3 kD
9. A peptide, 1.5 kD
10. Uracil, 120

## Proteins Separations at very low salt concentration

In separation of positively charged proteins, a high concentration of salt is needed to shield the strong electrostatic interaction between the silica packing and the proteins. For example, cytochrome C, a highly positively charged protein (pI=9.6) is very difficult to be eluted at a low salt concentration, such as 50 mM phosphate buffer at pH 7.0. The need for high concentration salt to elute proteins greatly limits the applications. Sepax designed a special *Nanofilm* SEC phase that could well elute positively

charged proteins. As shown in Figure 10, a specially designed *Nanofilm* SEC-500 eluted cytochrome C at 50 mM, phosphate buffer at pH 7.0, which could not be done with any other silica based SEC phases. Such specially designed *Nanofilm* SEC phases can expand size exclusion chromatography to a variety of new applications, such as separation of salt sensitive biological molecules, protein binding studies, and LC/MS detections.

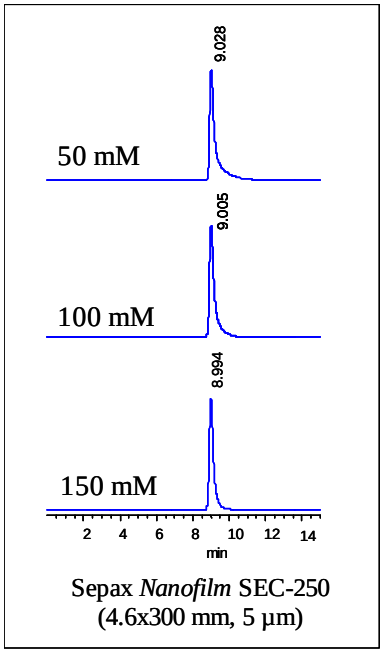



Figure 10. Cytochrome C elution profile with phosphate buffer at pH 7.0.
Column: *Nanofilm* SEC-250 (5 µm), 4.6x300 mm
Flow rate: 0.35 mL/min
Detection: UV 214 nm
Temperature: ambient (23 °C)
Injection volume: 5 µL
Sample: 1.0 mg/mL.

## Nanofilm SEC Applications

*Separation of proteins and peptides*

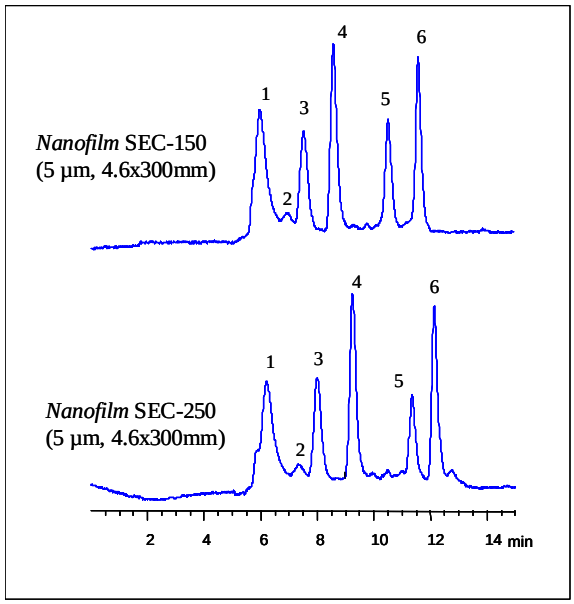



Figure 11. Separation of a mixture of proteins, a peptide and uracil by a Nanofilm SEC-150 and a Nanofilm SEC-250 column

Mobile phase: 0.15 M Phosphate buffer, pH 7.0
Flow rate: 0.35 mL/min
Detector: UV 280 nm
Injection volume: 5 µL
Sample: 1. Thyroglobulin (1.0 mg/mL), 670 kD
2. BSA dimer, 132 kD
3. BSA (1.0 mg/mL), 66 kD
4. Ribonuclease A (1.0 mg/mL), 13.7 kD)
5. A peptide (0.1 mg/mL), 1.5 kD
6. Uracil (0.02 mg/mL), 120

*Separation of horse serum at low salt concentration*

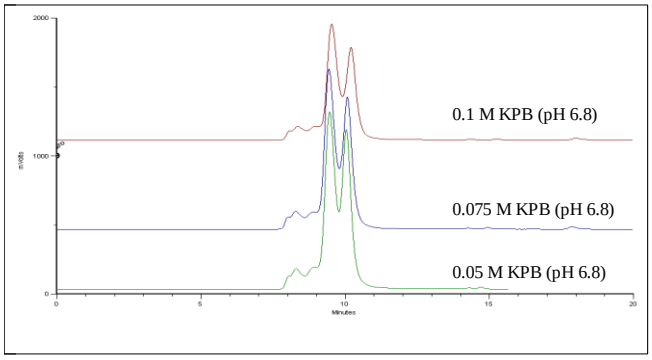



Figure 12. Separation of horse serum at various buffers with low salt concentrations

Column: Nanofilm SEC-150 (5µ, 4.6x300mm)
Mobile phase: phosphate buffer at pH 6.8
Flow rate: 0.25 mL/min
Injection volume: 1.0 µL
Detection: 280 nm
(Courtesy of Miyako Kawakatsu, M&S Instruments Inc.)

## Applications

- *Protein, nucleic acid and peptide separations*
- *Natural and synthetic polymer separations*
- *Protein binding studies*
- *Analytical, semi-preparative, and preparative separations*
- *Proteomics*
- *Special Applications for LC/MS*

## Nanofilm SEC Technical Specifications

| Phase | *Nanofilm* SEC-150 | *Nanofilm* SEC-250 | *Nanofilm* SEC-500 | *Nanofilm* SEC-1000 |
|---|---|---|---|---|
| Material | Nanometer, hydrophilic film bonded silica | Nanometer, hydrophilic film bonded silica | Nanometer, hydrophilic film bonded silica | Nanometer, hydrophilic film bonded silica |
| Particle size | 5, 10 µm | 5, 10 µm | 5, 10 µm | 5, 10 µm |
| Pore size (Å) | ~ 150 | ~ 250 | ~ 500 | ~ 1,000 |
| Protein MW range (native) | *200 - 750,000* | *1,500 – 1,250,000* | *15,000 - 2,500,000* | *50,000 - 5,000,000* |
| pH stability | 2 – 8.5 (pH 9.0 can be tolerated temporarily.) | 2 – 8.5 (pH 9.0 can be tolerated temporarily.) | 2 – 8.5 (pH 9.0 can be tolerated temporarily.) | 2 – 8.5 (pH 9.0 can be tolerated temporarily.) |
| Backpressure (psi for a 4.6x300 mm) | ~ 700 | ~ 700 | ~650 | ~650 |
| Maximum backpressure (psi) | ~ 3,500 | ~ 3,500 | ~ 3,000 | ~ 3,000 |
| Salt concentration range | 20 mM - 2.0 M | 20 mM - 2.0 M | 20 mM - 2.0 M | 20 mM - 2.0 M |
| Maximum temperature (°C) | ~ 80 | ~ 80 | ~ 80 | ~ 80 |