MacroCap SP

MacroCap™ SP is a cation exchanger designed for the purification of large biomolecules such as polyethylene glycol (PEG)-modified proteins (i.e., PEGylated proteins) that are intended for use as biopharmaceuticals.

PEGylation typically changes a native protein sample into a mixture of native protein and much larger-sized PEG-protein conjugates of varied PEG-to-protein mole ratios. Because PEG is neutral, such conjugates exhibit lower average surface charge. In post-PEGylation purification steps, these two factors (larger size and lower charge) adversely affect capacity and resolution (1). These factors also contribute to fouling of the medium, which reduces its lifetime. MacroCap SP (Fig 1) overcomes these issues.

MacroCap SP provides:

- High purity and yield of PEGylated proteins at high sample loads
- Good cleaning-in-place (CIP) stability for long medium lifetime and economical operation

Fig 1. MacroCap SP cation exchanger can be used to purify PEGylated and other large biomolecules to high purity and yield at high sample loads. Robust chemical stability helps ensure a long medium lifetime.

Medium characteristics

Designed to separate large biomolecules

MacroCap SP is based on GE Healthcare's proprietary media with mass transfer properties suitable for large biomolecules. The base matrix is highly porous, which gives high available surface area for adsorption of large molecules. Basic characteristics are shown in Table 1.

High purity and yield at high sample load

MacroCap SP is designed to separate PEGylated and other large biomolecules. It allows separation of mono- from oligo- and non-PEGylated proteins with high selectivity under high load conditions. Figure 2 shows the separation of cytochrome C modified with 20 000 M_r PEG on MacroCap SP, at a sample load of 6 mg protein/ml medium. The dynamic binding capacity ($Q_{B10\%}$) for mono-PEGylated cytochrome C was 3.8 mg/ml.

Since PEGylation generally involves pure native protein, the economic value of the product is typically very high. Thus good recovery of target PEGylated protein is of primary importance for overall productivity. The results show that based on absorbance, 99% of the mono-PEGylated protein could be recovered from MacroCap SP at a purity by size analysis of 93%.

The large pore size also makes MacroCap SP suitable for binding other large proteins. Figure 3 compares binding of IgM (M_r 750 000) to MacroCap SP and SP Sepharose™ High Performance.

Table 1. Key characteristics of MacroCap SP

Matrix	Cross-linked copolymer of allyl dextran and N,N-methylene bisacrylamide
Ion exchange type	Strong cation
Charged group	$-SO_3^-$
Total ionic capacity	0.10 to 0.13 mmol H^+/ml medium
Particle size[1]	50 µm (d_{50v})
Flow velocity	120 cm/h in BPG 300, 20-cm bed height or 70 cm/h in BPG 300, 30-cm bed height using process buffers with the same viscosity as water at < 3 bar (0.3 MPa)
Recommended separation range	a) Proteins in excess of 150 000 M_r b) Functionalized dextrans or PEGs greater than or equal to 20 000 M_r c) PEGylated proteins containing greater than or equal to 10 000 M_r of PEG (total) per conjugate.
pH stability[2]	
short-term	2 to 13
working	3 to 12
long-term	4 to 11
Storage temperature	4°C to 30°C
Chemical stability	All commonly-used aqueous buffers, 0.5 M NaOH, 0.1 M citric acid, 25% ethanol, 30% propanol, 30% methanol, 50% ethylene glycol, 1% Tween™-20, and 1% SDS.

[1] d_{50v} is the medium particle size of the cumulative volume distribution.

[2] Short-term pH: pH interval where the medium can be subjected to cleaning-in-place or sanitization-in-place (accumulated 90 to 400 h at room temperature) without significant change in function.

Working pH: pH interval where the medium binds protein as intended or that needed for elution without adverse long-term effect.

Long-term pH: pH interval where the medium can be operated without significant change in function.

Good chemical stability and long medium lifetime

MacroCap SP has good chemical stability, which allows CIP to be performed both at acidic and alkaline conditions. The hydrophilic nature of the MacroCap SP base matrix reduces nonspecific binding and reduces fouling issues that may be experienced with more hydrophobic base matrices. Together these features assure long medium lifetime.

Figure 4 shows that the selectivity and binding capacity of the medium for RNase A, cytochrome C, and lysozyme were unchanged after 30 cycles of CIP involving acidic and alkaline conditions in each cycle.

Meets industrial needs

MacroCap SP is a GE Healthcare BioProcess™ medium, specifically designed to meet the demands of industrial biotechnology. This means that the medium is scalable from laboratory to production, is produced with validated manufacturing procedures, and can withstand standard CIP and sanitization-in-place procedures. In addition, BioProcess media are supported with regulatory support files and comprehensive documentation, as well as security of supply service.

Column: Tricorn 5/100 (bed height 107 mm; column volume (CV) 2.1 ml) packed with MacroCap SP
Sample: Cytochrome C modified with 20 000 M_r PEG
Sample load: 6 mg total protein per ml medium
Buffer 1: 0.02 M sodium phosphate, pH 6.8
Buffer 2: Buffer A + 0.4 M sodium chloride
Flow rate: 0.2 ml/min (61 cm/h)
Gradient: 0 to 100% Buffer B in 20 CV
System: ÄKTAexplorer™ 10

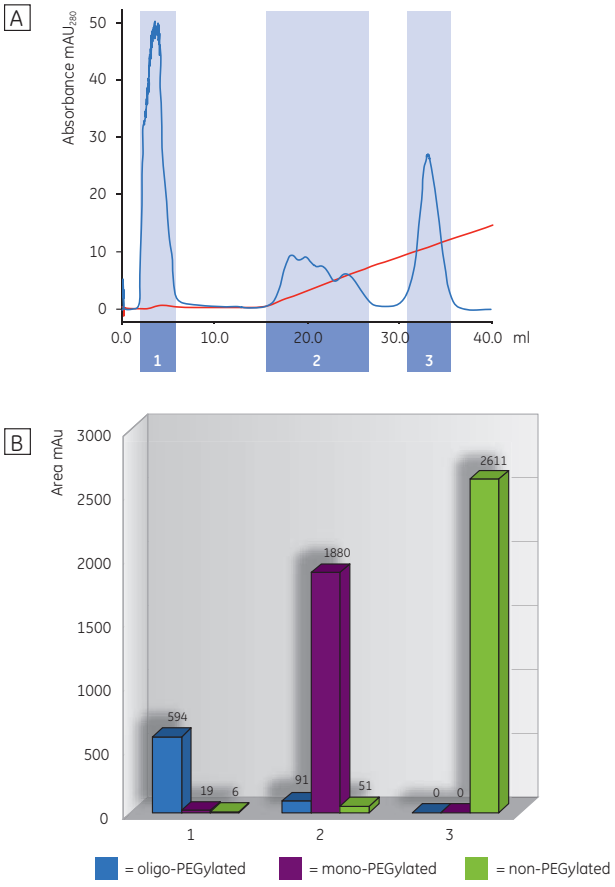

Fig 2. PEGylated cytochrome C[1] separated on MacroCap SP. **(A)** Chromatograms of the separation. Fractions that were pooled for the size exclusgion chromatography (SEC) analysis are indicated in blue and numbered. **(B)** Pooled fractions indicated in **(A)** were analzyed by SEC on Superdex™ 200 for the amounts of oligo-, mono-, and non-PEGylated proteins.

[1] Bovine cytochrome C (Sigma Aldrich, USA) covalently modified with monomethoxy-PEG 20 000 succinimidylproprionic acid (SPA) reagent (Nektar Therapeutics, USA).

Column: Tricorn 5/100 packed with either (A) MacroCap SP or (B) SP Sepharose High Performance (CV 2 ml)
Sample: IgM (human), 96% pure by HPLC
Sample load: 0.5 mg/ml medium
Buffer A: 100 mM sodium acetate, pH 4.75
Buffer B: Buffer A + 0.5 M sodium chloride
Flow rate: 0.3 ml/min (90 cm/h)
Gradient: 0 to 100% Buffer B in 20 CV
System: ÄKTAexplorer 10

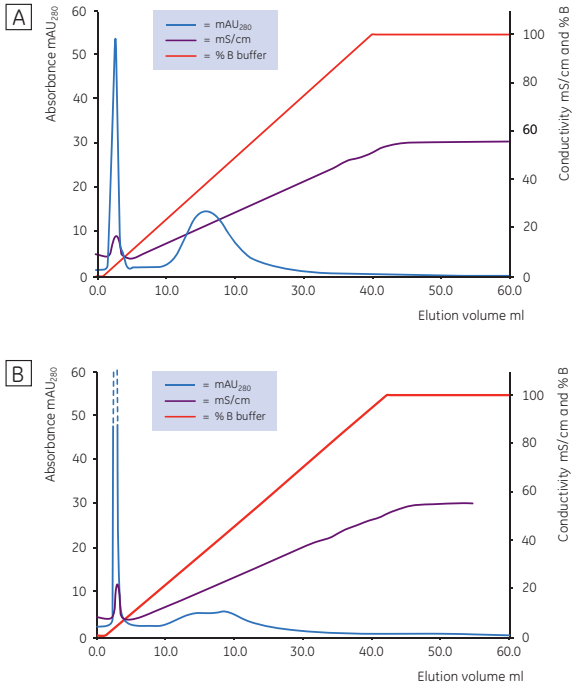

Fig 3. Binding and elution of a pure (96% by HPLC) sample of IgM (M_r = 750 000) (Sigma Aldrich, USA) on **(A)** MacroCap SP and **(B)** SP Sepharose High Performance. The large pore structure of MacroCap SP gives it a greater capability to bind large biomolecules.

Column: MacroCap SP in 10 mm i.d., 9 cm bed height column (CV 7.1 ml)
Sample: 0.5 mg/ml RNase A, 0.5 mg/ml cytochrome C and 0.5 mg/ml lysozyme
Sample load: 1 CV
Buffer A: 20 mM phosphate buffer at pH 6.8
Buffer B: Buffer A + 0.4 M NaCl
Flow rate: 1 ml/min (75 cm/h)
Gradient: 0% to 100% Buffer B in 15 CV
System: ÄKTAexplorer 100

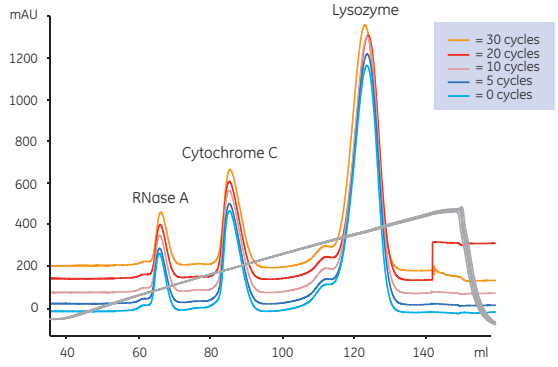

Fig 4. CIP study of MacroCap SP. The performance of MacroCap SP in the separation of RNase A, cytochrome C, and lysozyme was unaffected following 30 cycles of a CIP procedure. CIP conditions per cycle: 5 column volumes (CV) H_2O, 2 CV 0.5 M NaOH followed by 40 min static contact, 2 CV H_2O, 2 CV acidic solution (~pH 2) followed by 40 min static contact, 2 CV H_2O, 5 CV 0.5 M NaOH followed by 40 min static contact, and 5 CV H_2O.

Operation and method development for PEGylated proteins

How PEGylation affects protein purification

PEGylation changes a native protein into a much larger-sized PEG-protein conjugate of lower average surface charge. By weight, PEG polymers typically occupy over six times the hydration volume of globular proteins. Both effects (larger size and lower surface charge) increase with the degree of PEGylation. Consequently, the dynamic binding capacity (mg of protein per ml gel) of an ion exchange medium for PEGylated proteins, compared with native proteins, can decrease by a factor of about 10. In a typical ion exchange gradient run with a PEGylation reaction mixture, the products will elute in the following order: free PEG substances, oligo-PEGylated proteins, mono-PEGylated proteins, and non-PEGylated proteins.

During sample loading, proteins with higher surface charge will often displace those with lower charge, and non-PEGylated proteins exhibit some capacity to displace PEGylated proteins at higher sample loading. Ease of displacement is related to the degree of PEGylation and other factors such as solution conductivity. As a result, greater loading may contribute to higher target purity in such situations.

The above factors suggest that loading PEGylation reaction mixtures using buffers with a conductivity that promotes flow through of non-target PEGylated proteins, and optimal binding of target and non-PEGylated proteins, will make better use of column capacity.

Development and optimization

The aim of designing and optimizing a method for the separation of large biomolecules, such as PEGylated proteins, is to ensure high and consistent binding capacity and selectivity, enabling robust and scalable operation.

MacroCap SP operates as a normal cation exchanger. It has been designed for large-scale operation, and allows flow velocities of 120 cm/h at 20-cm bed height in a 30 cm i.d. column (70 cm/h at 30-cm bed height). These are common operating flow velocities for processing of large, slowly diffusing biomolecules. A faster flow may be possible with lower bed heights. Note, however, that residence times of 6 to 15 min are recommended to fully exploit the properties of the medium, mainly because larger molecules diffuse slower and require more time to bind to the medium.

Method development can be done in small columns, such as Tricorn™ columns. A detailed purification protocol is given in the instruction manual. In addition to reducing protein charge density, PEGylation may alter protein pI by 1 unit or more. As a result it is suggested to experiment with adsorption at lower than normal pH and conductivity.

The difference in performance between various ion exchange media for large biomolecules may not be evident at low sample loadings. However, at high sample loadings, the greater capacity and resolution properties of MacroCap SP become evident, as do reduced fouling and other properties that make MacroCap SP the medium of choice to purify large biomolecules, including PEGylated proteins.

Scale-up

Laboratory methods can be scaled up using small-diameter columns and working up to the final intended large-scale column height and linear flow rate (residence time). Scale-up can be continued by increasing column diameter. Table 1 lists flow rates and other recommendations to consider when scaling up the use of MacroCap SP. Reducing bed height will often allow for the use of a significantly greater flow rate.

Cleaning-in-place

Stability studies have demonstrated that MacroCap SP resists harsh CIP conditions at both low and high pH. Note that specific CIP protocols should be developed according to the feedstock applied and other related operating conditions.

Summary

MacroCap SP is a cation exchanger designed to purify PEGylated and other large biomolecules, at high sample load. Mono-PEGylated proteins can be separated to high purity from oligo-PEGylated and non-PEGylated proteins in a single run. Good CIP stability allows long medium lifetime and eliminates fouling issues that may be experienced with more hydrophobic base matrices.

Reference

1. Fee, C. J. and Van Alstine, J. M. PEG-proteins: Reaction engineering and separation issues. *Chem. Eng. Sci.* **61**, 924–939 (2006).

Ordering information

Product	Pack size[1]	Code number
MacroCap SP	25 ml	17-5440-10
MacroCap SP	100 ml	17-5440-01
MacroCap SP	1 l	17-5440-02
MacroCap SP	5 l	17-5440-03

[1] Larger quantities are available. Please contact GE Healthcare for more information.

Literature	Code no.
Ion Exchange Chromatography & Chromatofocusing: Principles and Methods	18-0004-21

www.gehealthcare.com/protein-purification
www.gehealthcare.com

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28-4005-84 AA 01/2006