Amsphere™

PURIFICATION OF ANTIBODY FRAGMENTS AND SINGLE DOMAIN ANTIBODIES WITH AMSPHERE A3 PROTEIN A RESIN

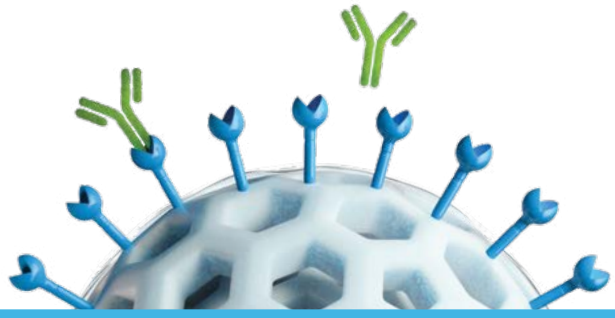
Besides the use for Fc containing antibodies and constructs, the Protein A resin Amsphere™ A3 can be used for the purification of antibody fragment formats, like the VHH single domain antibodies (sdAbs), constructs containing a VH3 domain like Fragment Antigen-Binding molecules (Fabs) and different fusion proteins such as single-chain variable fragment (scFvs).

This application note contains key information regarding the use of Amsphere™ A3 for the affinity purification of these VHH sdAbs and antibody fragments that contain a VH domain, but not an Fc region. Recommendations for experimental protocols are given. The Amsphere™ A3 Instruction Manual provides more comprehensive information on the general use of this resin.

Background

Due to the lack of an Fc region, antibody fragments cannot be captured with most of the caustic-stable Protein A affinity ligands. However, the Amsphere™ A3 Protein A ligand exhibits a high affinity for VHH single domain antibodies and Fabs. The crystal structure of a VHH-PrA complex was resolved and the amino acids in the VHH involved in the binding were identified. All these residues are conserved in the VH3 domain of the Fab region of a classical antibody. This strongly suggests a similar binding mechanism for both VHH and VH domains.

For more details, please consult our white paper, entitled “Purification of antibody fragments and single domain antibodies with Amsphere™ A3 Protein A resin” on the JSR Life Sciences website (www.jsrlifesciences.com).



Amsphere™ A3 Construct

Amsphere A3 is a new protein A resin designed with a surface modified base bead and novel, alkali-resistant ligand.

Protein A ligand

- High DBC via controlled conformation and orientation
- High alkaline stability from protein engineering

Surface modification

- Low HCP levels by surface hydrophilization

Base bead formulation

- High DBC at high flow rate
- Excellent pressure and flow properties via rigid crosslinking

Recommendations on Purification Protocol

A standard Protein A affinity chromatography protocol, used for mAbs, can be used for antibody fragments as well. Some attention points for antibody fragment affinity purification with Amsphere™ A3 are mentioned in this chapter.

Equilibration and Feed Loading

For equilibration, a common buffer at neutral pH can be used, e.g. sodium phosphate or Tris-HCl. If the packed column is to be used after long-term storage, it is recommended that a blank run including CIP is performed prior to use.

For a number of VH and VHH sdAbs, a variety of binding conditions was tested. No significant differences in DBC were found for a range of feed pH (6.8–7.6), conductivity (5–45 mS/cm), residence times (2–6 min) and titers (0.1–4.2 g/L). The use of a reducing agent (100mM thioglycerol) in the HCCF also did not have an impact on the DBC.

The binding of a Fab fragment, created by papain digest of Herceptin, was found to be salt dependent. *It is therefore recommended to use low conductivity conditions (< 5.0 mS/cm) for initial binding studies of VH domain related targets, and scout higher conductivity conditions afterwards.*

Wash Steps

After binding of the fragments has been confirmed as described in the previous chapter, the wash steps can be optimized to further improve HCP (and sometimes DNA) clearance. For VHH sdAbs, a high salt wash step (0.5–1.0 M NaCl) can be used, without any product loss.

Elution

The product of interest bound to the resin can be eluted with acidic buffers (pH 2.7–4.0). We have obtained recoveries over 95% in elution volumes ≤ 3 CV for glycin-HCl (100mM; pH 2.7), sodium citrate (50mM; pH 3.2) and sodium acetate (50mM; pH 3.0) buffers.

To determine the optimum pH for elution, a linear pH gradient study may need to be performed at small scale.

Acidic Strip, Cleaning In Place (CIP) and Sanitization

After elution, the column can be further washed with acidic buffers to remove residual proteins or impurities. Acids such as acetic acid, hydrochloric acid, or phosphoric acid (pH 1.5–3.0; 20–200 mM) can also be used.

For CIP of Amsphere™ A3, caustic solutions such as 0.1–0.5 M NaOH can be used. Other buffers containing widely-used chemical components such as surfactants, organic solvents, and organic/inorganic salts can also be used. Although Amsphere™ A3 has a high caustic stability, a long contact time with alkaline buffers will cause the DBC to gradually decrease. Equilibrate the column immediately after CIP.

Table 1 gives an overview of chemical stability results related to exposure times of Amsphere™ A3 in different sodium hydroxide concentrations. Depending on the desired CIP and sanitization regime, the resin lifetime in number of cycles can be computed from the data in the table. The 80% DBC retention is given as it is often used as an endpoint for determining useful resin lifetime.

Table 1: Caustic stability data for Amsphere™ A3. Exposure times for different sodium hydroxide concentrations are shown after which the DBC for polyclonal IgG at 4 min residence time is still 90 and 80% of the starting value.

NaOH Concentration (mol/L)	Exposure Time at 22°C (hrs)	
	At 90% DBC Retention	At 80% DBC Retention
0.1	106	162
0.2	37	68
0.5	18	27

Storage

Amsphere™ A3 is shipped in a 50mM sodium phosphate buffer in 16% (v/v) ethanol. It should be stored at 2–8°C and never frozen. To store a column packed with Amsphere™ A3, wash the column with buffer at neutral pH containing 16–20% (v/v) ethanol and store at 2–8°C. A 2% benzyl alcohol solution can also be used instead of ethanol.

Expected Results – Examples

For VHH sdAbs, more than 100 molecules have been screened, of which 99% showed binding to Amsphere™ A3.

For VH binding, experimental data and the literature indicate that significant binding of Protein A to VH is restricted to molecules of the human VH3 subfamily, with no examples from any of the other gene families.

DBC

Table 2 shows examples of DBC values obtained for antibody fragments with different degrees of complexity in terms of number of antibody domains that are linked. Because these molecules have a smaller hydrodynamic radius than mAbs, they diffuse faster in and out of the particle pores. Therefore, for the smaller molecules, the DBC does not increase anymore when increasing the residence time in the column above 2.0 min.

Table 2: Expected range of DBC values of Amsphere™ A3 for antibody fragments and sdAbs with different molecular weight.

Target Molecule Type	MW (kDa)	Highest DBC Observed (g/L)	Residence Time (min)
Monovalent VH/VHH	12–20	30	2
Bivalent, trivalent and tetravalent VH/VHH	30–60	35	2
Higher complexity of fused Ab domains	60–100	50	4

Compared to full size mAbs, the capacity values on a mass-per-resin volume (g/L) basis shown for monovalent and bivalent VHHs are about 2–3 times lower. On a molar basis however, it can be expected that about 3 to 4 times more monovalent VHHs bind per multimeric Protein A ligand than for mAbs.

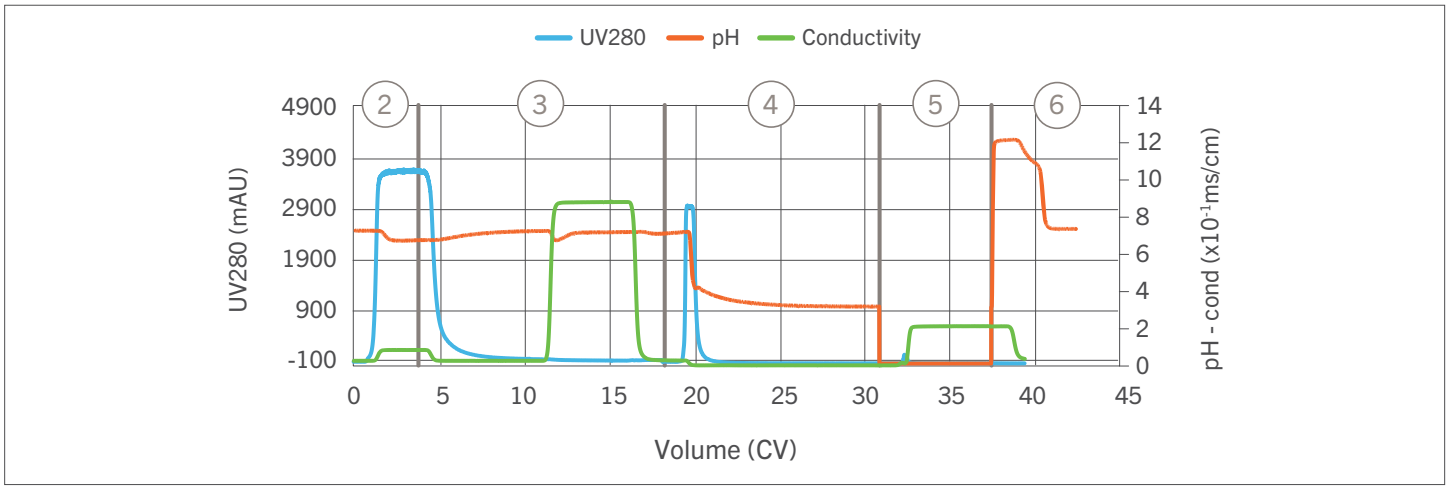
Impurity Clearance

Figure 2 and table 4 show an example of the chromatographic performance of a batch purification run of a VHH with Amsphere™ A3. The conditions used for this purification are summarized in table 3.

Table 3: Protocol used for purification of a VHH sdAb to Amsphere A3. (*): HCCF = clarified feed VHH sdAb (Pichia Pastoris supernatant) with conc. 4.1 g/l – pH 6.9 and 8.8 mS/cm. (**): pH meter offline.

	Step	Solution — Buffer	Flow (cm/h)	Res. Time (min)	Length (CV)
1	Equilibration	20mM sodium phosphate pH 7.5	300	1.0	5.0
2	Load	VHH HCCF (*)	100	3.0	3.2
3	Wash 1	20mM sodium phosphate pH 7.5			7.0
	Wash 2	20mM sodium phosphate; 1.0M NaCl ; pH 7.5			5.0
	Wash 3	20mM sodium phosphate pH 7.5			3.0
4	Elution	50mM sodium acetate pH 3.0			12.0
5 (**)	CIP part 1	0.1M NaOH (elution buffer replacement)	150	2.0	3.0
	CIP part 2	0.1M NaOH (15 min contact time)	55	5.5	3.0
6	Equilibration	20mM sodium phosphate pH 7.5	300	1.0	5.0

Figure 1: Purification of a VHH sdAb with Amsphere A3. **Blue:** UV280 absorbance. **Green:** conductivity (mS/cm). **Orange:** pH. Experiments were performed on an ÄKTA avant™ 25. A Pichia pastoris 2nd Generation HCP ELISA kit (Cygnus Technologies, cat no F640) was used for HCP measurement.



The results shown in table 4 demonstrate a low elution volume with good protein recovery and HCP clearance. Removal of HCP and DNA is similar to the known values of 2 – 3 LRVs from IgG affinity capture.

Table 4: Purification results of the run conditions in table 3, for which the chromatogram is depicted in figure 1.

Parameter	Value	Unit
Column load	13	g/L
Elution volume	1.6	CV
Recovery	98	%
HCP LRV	2.7	Log

Abbreviations

Ab: Antibody
CIP: Cleaning In Place
CV: Column Volume
DBC: Dynamic Binding Capacity
Fab: Fragment Antigen-Binding
Fc: Fragment crystallizable
HCCF: Harvested Cell Culture Fluid
HCP: Host Cell Protein
IgG: Immunoglobulin G
kDa: kilodalton
LRV: Log Reduction Value
mAb: Monoclonal Antibody
MW: Molecular Weight
scFv: single-chain variable fragment
sdAb: Single Domain Antibody
VH: Variable domain of the Heavy chain
VH3: Variable domain of the Heavy chain of the gene family 3
VHH: Variable domain of the Heavy chain of a (camelid) Heavy chain antibody

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