Functionalized Nanoparticle Crosslinking for Enhanced Affinity Precipitation of Monoclonal Antibodies

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Abstract

Recent advancements in upstream therapeutic monoclonal antibody (mAb) production have posed significant challenges to the downstream purification platform. Protein A affinity chromatography has been identified as a potential process bottleneck due to limitations in throughput, scale-up, and cost. Affinity precipitation is a promising non-chromatographic alternative because it combines the selectivity of an affinity ligand with the operational benefits of conventional precipitation. After binding to a target protein in solution, phase transition is induced through crosslinking with multivalent affinity ligands (primary effect; multimeric protein only) or using stimuli-responsive affinity ligands (secondary effect; monomer or multimer). Primary effect affinity precipitation is not widely used due to low precipitation yields, but there are several examples of mAb purification using protein A ligands conjugated to a responsive domain of synthetic or natural origin.

Our group has demonstrated the feasibility of using a recombinant antibody binding domain (Z-domain) genetically fused to a temperature responsive elastin-like polypeptide (ELP) biopolymer (Z-ELP) for mAb capture and precipitation. However, the high temperature and salt concentrations and the heating/cooling steps required for reversible Z-ELP phase transition impacted antibody stability and operational efficiency. We proposed a two-part solution: Increase the affinity ligand multivalency and

dimension by scaffolding Z-ELP to a nanoparticle using site-specific conjugation. We suspected that this would enable primary effect mAb-nanoparticle crosslinking, while also improving ELP precipitation by enlarging the scale of aggregate formation.

The first objective was to engineer and characterize the functionalization of Z-ELP to a self-assembled, thermostable E2 nanocage (Z-ELP-E2), derived from *Bacillus stearothermophilus* using Staphylococcal Sortase A bioconjugation. We demonstrated a significant decrease in ELP transition temperature and an increase in aggregate size upon antibody binding at isothermal solution conditions. We concluded this was due to multivalent antibody-Z-ELP-E2 interactions that nucleated into a crosslinked network. The second objective was to develop an affinity precipitation process capable of purifying industrial monoclonal antibodies at ambient temperature with minimal added salt. We discovered that the nanoparticles rapidly aggregated upon mixing with mAb cell culture harvests due to multivalent crosslinking into large, insoluble particles. After optimization of key process steps, the affinity precipitation mAb yield and impurity clearance was found to meet or exceed Protein A chromatography performance.

Our third objective was to establish a platform that can be applied to any target mAb or F_c-containing protein with minimal optimization of process parameters. Z-ELP-E2 affinity precipitation was evaluated using four industrial mAbs (mAbs A–D) and one Fc fusion protein (Fc A) with different molecular properties. For all molecules, a molar binding ratio of 3:1 Z:mAb was sufficient to precipitate > 95% mAb in solution at ambient temperature and without added salt. After centrifugation, the pelleted mAb-nanoparticle complex remained insoluble, and was capable of being washed at pH ≥ 5 and eluted at pH < 4 with > 90% mAb recovery for all molecules. The target proteins were purified using optimal process conditions and > 94% yield and > 97% monomer content were obtained. mAb A–D purification resulted in a reduction of 99.9% host cell protein and > 99.99% DNA from the cell culture harvests. However, Z-ELP-E2 regeneration yields of < 90 % limited the potential for reuse in subsequent purification cycles.

We suspected the regeneration yield may be improved by increasing the ligation density of Z-ELP on the E2 nanocage. Low conjugation efficiency was observed for the Sortase A functionalized

nanoparticles due to the reversible nature of enzymatic Sortase A ligation. A 5-fold molar excess Z-ELP reactant was required to drive the reaction to a maximum conversion of ~50%. In our fourth objective, we report an improved conjugation system using the split SpyTag/SpyCatcher isopeptide bond formation between SpyTag-E2 and Z-ELP-SpyCatcher fusion proteins. E2 ligation efficiencies exceeding 90% enabled the facile production of nanocages at any target Z-ELP density for tunable aggregation and mAb binding properties. 100% ligation Z-ELP_{80}-Spy-E2 nanocages were capable of selective precipitation using 0.1 M ammonium sulfate at 23ºC and improved the nanocage regeneration recovery to > 90%.

The fifth objective was to apply mAb-nanoparticle crosslinking to quantify mAb concentration in cell culture harvests. Existing mAb titer assays typically require expensive equipment or are limited by low-throughput or complicated protocols. We developed a quick and cost-effective alternative assay by measuring crosslinking-dependent turbidity after mixing 100% ligation Z-ELP_{80}-Spy-E2 nanoparticles with a mAb cell culture sample. A simple logarithmic regression was found to fit ($R^2 = 0.99$) the turbidity data for mAb concentrations between 100-1000 µg/mL. The optimized assay procedure was validated using two industrial mAb cell culture harvests and a bridging study with Octet RED96e biolayer interferometry confirmed accurate and reproduceable results.

Our final objective and aim of future work is to generalize the nanoparticle crosslinking strategy for the turbidity-based detection or purification of any target protein (monomer or multimer) using functionalized nanobody-ELP affinity ligands. Nanobodies are small, single-domain antibody fragments that can be engineered to bind to target proteins with high specificity. In addition, multiple nanobodies with orthogonal binding motifs on the same target can be identified through common screening methods. We hypothesized that the conjugation of two or more orthogonal binding nanobodies may induce crosslinking with monomeric proteins. We established proof of concept monomeric protein-nanoparticle crosslinking and turbidity-based quantification using a model green fluorescent protein (GFP) and two orthogonal GFP nanobody-ELP_{80}-SpyCatcher functionalized SpyTag-E2 nanoparticles.