SELECTIVE EXTRACTION OF RECOMBINANT PROTEINS BY MULTIPLE-AFFINITY TWO-PHASE PARTITIONING IN MICROCHANNELS

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ABSTRACT

We have demonstrated purification of proteins in a simple aqueous two-phase extraction process in a microfluidic device. The laminar flows inherent to microchannels allows us to perform a binary split of a complex cell lysate sample, in an open channel with no chromatography support and no moving parts. This mild process allows recovery of functional proteins with a modest increase in purity. Aromatic-rich fusion tags are used to drive partitioning of enzymes in a generic PEG-salt two-phase system. Addition of affinity ligands to the PEG phase allows us to exploit other popular fusion tags, such as polyhistidine tags and GST-tags.

KEYWORDS: Two-phase flow, aqueous two-phase systems, metal affinity partitioning, proteomics

INTRODUCTION

High-throughput protein expression experiments often require only microgram amounts of proteins, but conventional bench-scale protein purification techniques are typically inefficient when dealing with small amounts of starting material [1]. Microfluidic techniques are inherently scaled for the purification of small protein samples, along with the possibility of highly parallel, integrated operations.

We have previously described our efforts to create a simple process for selective extraction of proteins from cell lysate, using short, aromatic-rich tags to drive partitioning towards a PEG-rich phase of an aqueous two-phase system (ATPS). Here, we describe our advances in micro-preparative aqueous two-phase extraction of proteins on a chip, incorporating affinity ligands for common protein fusion tags into our two-phase system to increase the range of protein targets that can be selectively purified. Introducing affinity ligands such as hydrophobic moieties, dyes, or chelated metals into the phase system has long been known to improve the specificity of ATPS separations, although the cost of PEG modified with affinity ligands often precludes their use at a large scale. At the microscale, however, reagent cost is less significant in the overall economics of the process, and multiple types of affinity interactions can be considered for high-throughput protein purification.

THEORY

A simple schematic of our extraction process is illustrated in Figure 1. A sample stream is hydrodynamically focused between a PEG-rich and salt-rich feed streams; the overall composition of the three streams would lie within the two-phase region of the ATPS phase diagram. Two-phase laminar flow is established within ~10 mm of the focusing region. The channel is long relative to its width ($L/d \sim 2700$), allowing significant diffusional spreading of analytes away from the interface.

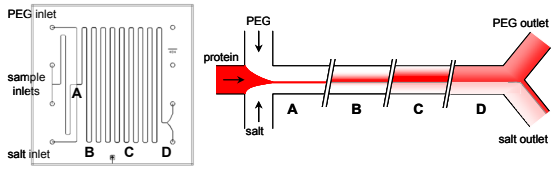

Figure 1: Channel layout for a continuous laminar flow aqueous two-phase extraction in a long, serpentine channel (484 mm long, 180 µm max width, 50 µm deep), illustrating partitioning and spreading of an analyte across the width of the channel.

There is a roughly 8-fold difference in viscosity between the two phases, resulting in a substantial velocity difference in the two phases. Reynolds numbers are calculated on both sides of the interface: $Re_{PEG} \sim 0.003$, while $Re_{salt} \sim 0.2$ for typical operations. Assuming that the diffusion coefficient of a typical analyte varies with the inverse of solution viscosity, the Peclet number for mass transfer is nearly identical on both sides of the interface. For a small protein with $D = 10^{-6}$ cm^2/s, $Pe_{PEG} \approx Pe_{salt} \sim 5000$, suggesting that even with $L/d \sim 2700$, the protein will diffuse significantly but not completely across the channel. The Peclet number for the PEG and salt themselves are likely still on the order of 1000, indicating that there is likely insufficient residence time for complete equilibration between the two phase forming streams, which are initially far from equilibrium with one another.

EXPERIMENTAL

Fluorescent proteins with polyhistidine tags (H_6-DsRedExpress and H_6-AcGFP1) were purchased from Clontech. Recombinant Glutathione-S-Transferase (GST) from *S. japonicum* was purchased from Genscript. Other recombinant proteins were produced using standard molecular biological protocols, as described previously.

Copper-chelating PEG (PEG-IDA-Cu^{++}) and PEG-glutatione conjugates were synthesized using variations of previously reported protocols. The "standard" PEG-rich and salt-rich feed streams consisted of 35 wt% PEG-4000 with 1.5 wt% potassium phosphate, pH 8, and 16 wt% potassium phosphate, pH 8, respectively. For ligand-modified phase systems, some portion of the PEG-4000 was replaced with ligand bearing PEG, such that the overall PEG concentration was maintained at 35 wt%. Microchannel fabrication and chip operation were as described previously.

RESULTS AND DISCUSSION

In our previous work, short, aromatic-rich partition tags such as Y_3P_2 dramatically improved partitioning of recombinant proteins in a PEG-salt ATPS, and allowed substantially improved recovery of functional green fluorescent protein as well as the enzyme GST. Although powerful, the partition tags are largely unknown to molecular biologists, and require significant validation to demonstrate that they have minimal impact on protein expression, stability, and function.

In this work, we extended our extraction process to explit ubiquitous polyhistidine tag and GST-tag. To this end, we synthesized metal-chelating PEG (PEG-IDA-Cu^{++}), as well as a PEG-glutathione conjugate. The metal-chelating PEG has a dramatic effect on the partitioning of polyhistidine-tagged proteins, which was visual-

ized during chip experiments with the autofluorescent proteins DsRedExpress (illustrated in Figure 2), and AcGFP1 (data not shown). It was expected that the combination of both metal affinity partitioning along with an aromatic-rich partition tag would have an even more dramatic effect than either tag individually, although testing with an AcGFP1 variant ($H_6Y_3P_2$-AcGFP1) did not bear this out.

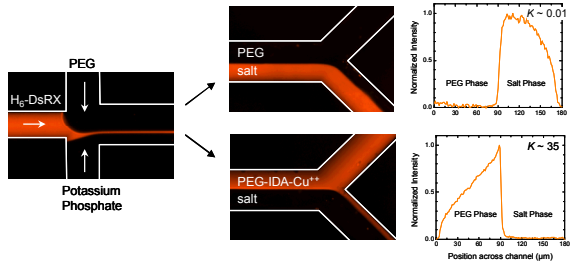

Figure 2: Fluorescent micrographs illustrating metal affinity partitioning in a microchannel. Recombinant DsRedExpress, an autofluorescent protein with a polyhistidine tag, partitions strongly to the salt-rich phase of a standard PEG-4000/potassium phosphate two-phase system. This behavior is reversed upon adding 10% PEG-IDA-Cu^{++} to the PEG inlet stream.

Testing with the PEG-glutatione conjugate is ongoing. The wild-type GST inherently partitions very strongly to the salt-rich phase, such that essentially no GST activity can be measured in the PEG-rich phase following extraction. Upon addition of varying amounts of PEG-glutathione to the PEG-rich phase, the GST activity measured in the PEG-rich phase increased in a linear fashion, up to about 20% PEG-GSH (as a fraction of total PEG), although the overall partition coefficient is still less than 0.1. Since the GST tag itself seems to strongly drive partitioning away from the PEG-rich phase, it may be rather useful in alternate strategies, in which a desired protein is to be recovered in the salt-rich phase.

CONCLUSIONS

Addition of metal affinity interactions to aqueous two-phase partitioning extends the range of targets that can be selectively purified using our microscale fractionation process. Glutathione affinity partitioning has thus far been less effective than either metal affinity partitioning or aromatic-rich partitioning, and testing is ongoing to develop effective chip-based strategies for high-throughput purification of proteins based on microfluidic extraction.

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