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Increased Microdialysis Recovery of Large Molecular Weight Analytes via Ultrafiltration

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Increased Microdialysis Recovery of Large Molecular Weight Analytes via Ultrafiltration

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I. Abstract

Microdialysis is a sampling method based on the passive diffusion of solutes across a semi-permeable hollow-fiber membrane that is driven by a concentration gradient. The membrane has a defined molecular weight cutoff, which causes larger molecular solutes such as proteins with molecular weights of approximately 8-80 kDa to have low recoveries. The purpose of this research is to utilize ultrafiltration across the membrane through push-pull and vacuum ultrafiltration methods as a means to increase recovery of large molecular weight analyte. These experiments were carried out using Methyl Orange (MO), Fluorescein Isothiocyanate 4 kDa (FITC-4), and Fluorescein Isothiocyanate 40 kDa (FITC-40). In comparison to conventional microdialysis, using push-pull methods (1 uL/min push with 1, 2, 3, 4, 5, and 8 uL/min pull, respectively) increased MO recovery up to 12%, FITC-4 recovery up to 47%, and FITC-40 recovery up to 37%. In comparison to conventional microdialysis, vacuum ultrafiltration methods (0 uL/min push with 1, 2, 3, 4, 5, and 8 uL/min pull, respectively) increased MO recovery up to 30%, FITC-4 recovery up to 64%, and FITC-40 recovery up to 87%.

II. Introduction

A. Microdialysis Sampling Overview

Microdialysis is a sampling method based on the passive diffusion of solutes across a semi-permeable hollow-fiber membrane that is driven by a concentration gradient. Because of microdialysis probes' small length and width (10mm length by 200-500 μm width), they are useful in many cases that require a small apparatus for sampling, such as analyte recovery in the brain.[4] The molecular weight cutoff (MWCO) of these probes typically ranges between 5 and 100 kDa. Fluid is continuously perfused through the inlet tubing and analytes that are smaller than the membrane pores can diffuse into the inner membrane lumen to be carried out through the outlet tubing as shown in Figure 1. Since analytes larger than the probe MWCO are unable to diffuse across the membrane, relatively clean samples can be recovered.[6]

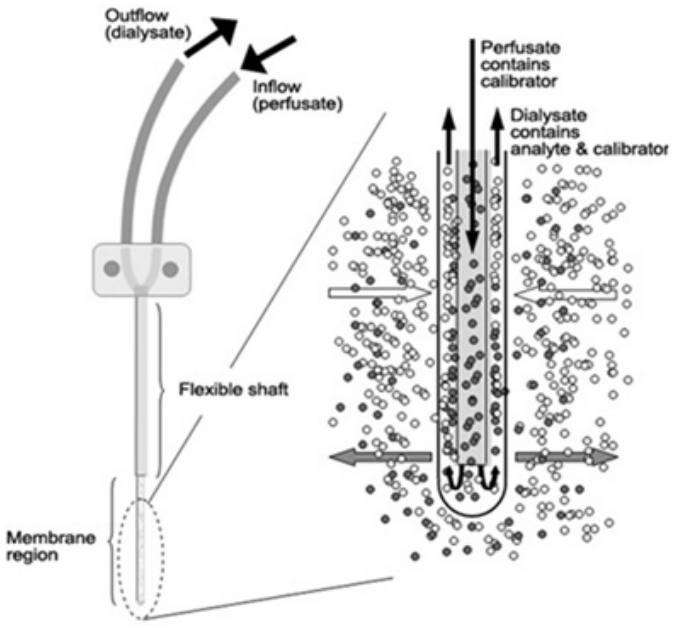

Figure 1. Diffusion across microdialysis probe.[2]

Microdialysis is often used for *in vivo* sample recovery because the tissue remains intact during sampling, which allows repeated sampling in animals without withdrawing blood. This often makes it possible for animals to act as their own controls.[7] It also allows continuous

monitoring of chemical changes within the extracellular fluid (ECF) at one implantation site.[4]

Microdialysis sampling has many clinical applications[8, 9] such as monitoring and studying cytokine activity in the brain.[10, 11] Cytokines are messenger proteins produced by various cells throughout the body that act as part of an immunological response. They work by binding to specific surface receptors on cells to initiate cascades of immune related responses that include inhibition, enhancement, or the alteration of cellular activity.[12] Microdialysis has been used to collect intravenous blood samples.[13] These samples are more stable because the enzymes that cause degradation of proteins are too large to diffuse across the membrane.[14] Microdialysis has also shown potential as a drug delivery system due to fluid loss across the membrane.[15, 16]

B. Theoretical Background

Microdialysis sampling is a pressure driven system which utilizes passive diffusion of solutes across a semi-permeable membrane. The pressure driving the system is exerted from the microdialysis pump (Figure 2) pushing the fluid from the syringe to the probe then out to the collection vial. Diffusion occurs across the membrane due to a concentration gradient. The solute concentration outside the probe is much greater than concentration within the probe, which facilitates mass transport.[3] Microdialysis membranes are generally made from polymers such as

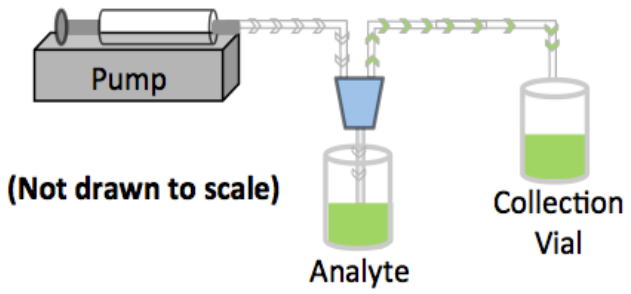

Figure 2. Microdialysis schematic.

polycarbonate-polyether (PC), copolymer cuprophan (CUP), polyacrylonitrile (PAN), or polyethersulfone (PES).[17] In this experiment, PES membranes with a 100 kDa MWCO will be used for sample recovery due to its availability.[6]

The performance of the microdialysis membrane with respect to mass transport is defined by the extraction efficiency (EE)[18],

$$\mathrm{EE} = \frac{C_{outlet} - C_{inlet}}{C_{sample} - C_{inlet}} \quad [1]$$

where C_{outlet} and C_{inlet} are the outlet and inlet analyte concentrations of the microdialysis probe, respectively, and C_{sample} is the analyte concentration far away from the probe in the external medium. Normally, the analyte concentration in the inlet fluid is zero, and EE becomes the relative recovery[6] (RR).

$$\mathrm{RR} = \frac{C_{outlet}}{C_{sample}} \quad [2]$$

RR is a function of the analyte mass transport properties through the sample solution, the membrane, the perfusion fluid[18], and is dependent on multiple factors such as flow rate of the perfusion fluid, membrane surface area and MWCO, analyte, and matrix properties of the sample media.[19]

C. Limitations

Microdialysis sampling provides many advantages and clinical applications, but it also possesses several limitations. Microdialysis can be a slow process, relatively speaking. Depending on time constraints and the analyte being tested, the process can take roughly 30 minutes to collect an adequate amount of sample for testing. This is, in part, due to the slow perfusion rates used for sampling – generally between 0.25-5.0 μL/min. Slower flow rates are used because RR of larger

molecular weight molecules decreases as flow rate increases[3, 20, 21] as shown in Figure 3. Increased flow rates can also increase fluid loss through the probe into the sample solution due to the increased pressure within the probe.[22]

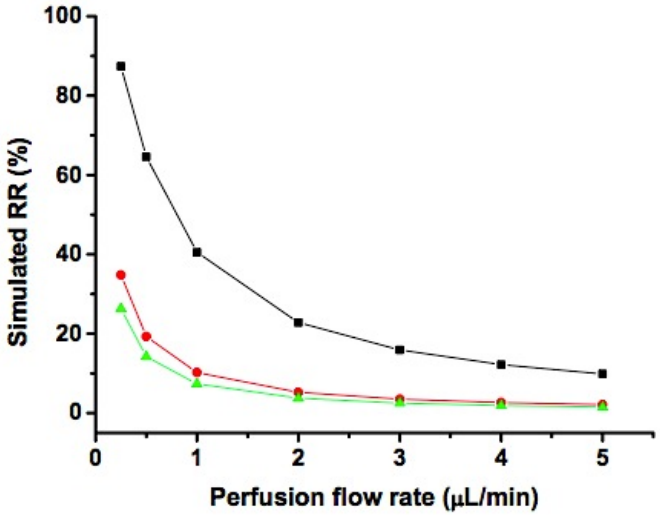

Figure 3. Simulated curves of RR as a function of flow rates using Bungay's model for glucose (black), insulin (red), and MCP-1 (green).[5]

Low RR of large molecular weight analyte is another limitation of microdialysis. This poses a problem when trying to recover proteins such as cytokines, which have a molecular weight of approximately 8-80 kDa and are usually found in the picomolar range in a biological system. Therefore, testing for the presence of these proteins requires sensitive methods for detection[6] or a relatively large sample volume.

Microdialysis sampling has been shown to yield higher recovery (>60%) for sampling low molecular weight analyte, such as glucose and urea, and is often used *in vivo*.[23-25] However, recovery using microdialysis is usually less than 100% because fluid is continuously flowing through the probe, meaning the perfusate and sample never fully reach a state of equilibrium[6] and the collected sample is diluted. Using higher flow rates reduces the residence time of the perfusate in the probe so analytes do not have enough time to diffuse across the membrane, which, therefore, decreases RR.[3] Variations and improvements have been made to the conventional microdialysis process in attempt to increase RR of analytes.

D. Variations of Microdialysis

Multiple variations of microdialysis have been tested to improve RR ranging from modifying the radius of the probe's inner cannula[26], utilizing multiple miniaturized probes at once[27], using affinity agents in the perfusate[28, 29], and increasing perfusate viscosity to decrease fluid loss.[30] Other modification techniques, which will be the primary focus of this experiment, that have been introduced to improve recovery are push-pull microdialysis and vacuum ultrafiltration.

i. Push-Pull Microdialysis

Push-pull microdialysis is a sampling technique in which a secondary pump is connected to the conventional microdialysis setup and set to "pull" sample as shown in Figure 4. This technique utilizes a pressure gradient to transport analyte across the microdialysis membrane. In conventional microdialysis, the syringe pump creates a positive pressure which forces perfusate through the system causing fluid loss into the sample (Figure 5a). When the secondary pump is connected, it creates a negative pressure environment in the outlet tubing that pulls analyte across the membrane, which eliminates fluid loss and increases RR (Figure 5b). By increasing the pull flow rate higher than the push flow rate (Figure 5c), the

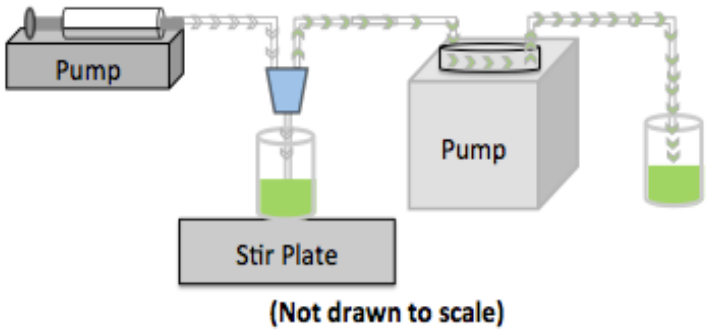

Figure 4. Push-pull schematic.

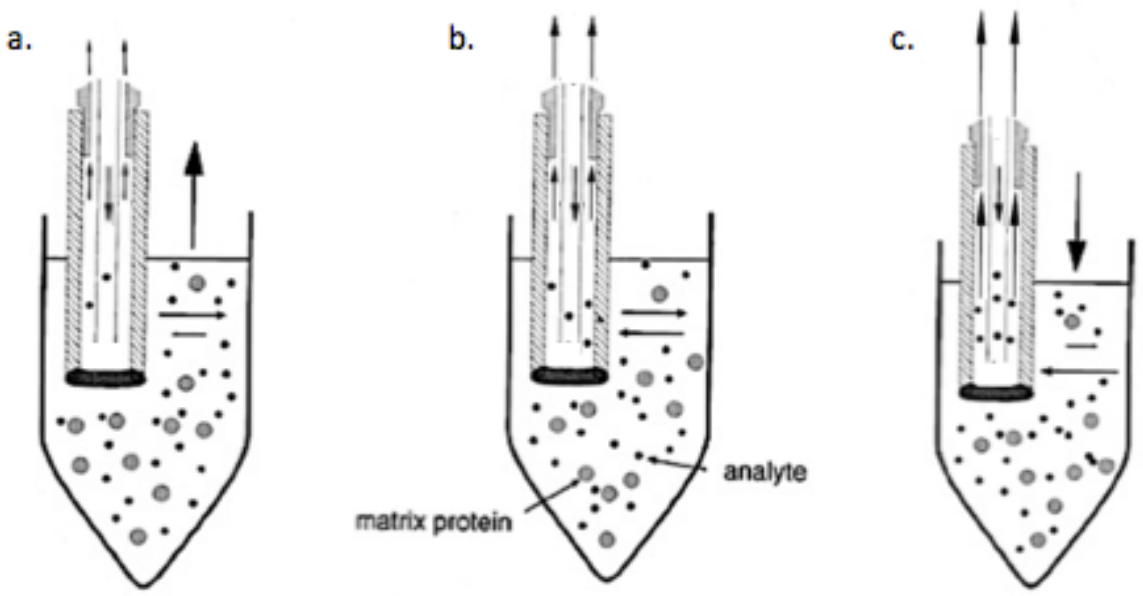

Figure 5. Image reprinted with permission from Kjellstrom et al.[1] **(a)** In conventional microdialysis, some of the perfusate will be lost into the sample. **(b)** In push-pull microdialysis using the same push and pull flow rates, the net flux across the membrane is zero. **(c)** Increasing the pull flow rate in push-pull microdialysis increases recovery and reduces the sample volume.

negative pressure in the outlet tubing is increased which causes an increase in analyte and fluid recovery.[1, 22]

ii. Vacuum Ultrafiltration

Ultrafiltration utilizes a pressure differential applied across a semipermeable membrane to transport analyte from the sample into the microdialysis probe.[7] Vacuum ultrafiltration uses the same setup show in Figure 4, but the inlet tube is sealed off so only the secondary pump is utilized to pull analyte across the membrane. Eliminating the perfusate prevents dilution of the recovered analyte, therefore increasing RR and fluid recovery (Figure 6).

Both push-pull microdialysis and vacuum ultrafiltration utilize ultrafiltration in their recovery mechanism. The purpose of this experiment is to determine which sampling technique yields higher RR of large molecular weight analyte.

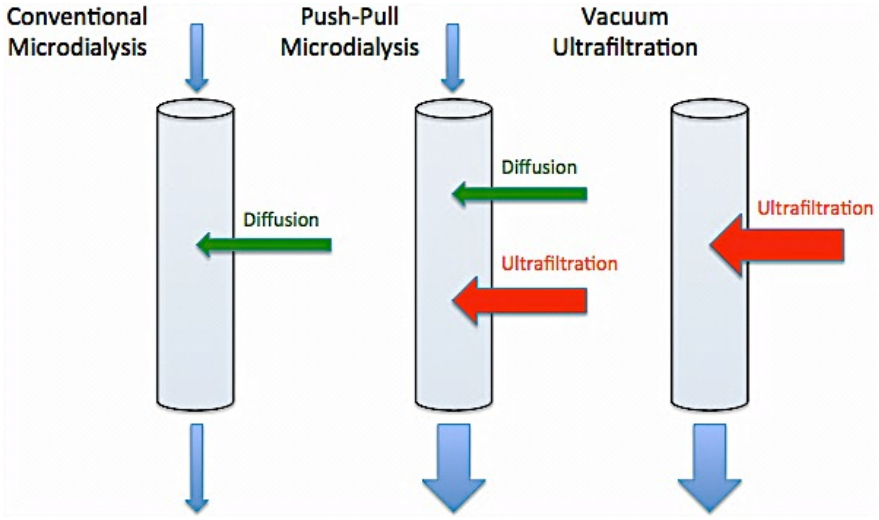

Figure 6. Schematic of mechanisms for conventional microdialysis, push-pull microdialysis, and vacuum ultrafiltration.

III. Materials and Methods

A. Chemicals and Instruments

Dextran 500, Methyl Orange (MO), Fluorescein Isothiocyanate 4 kDa (FITC-4), and Fluorescein Isothiocyanate 40 kDa (FITC-40) were purchased from Sigma-Aldrich (St. Louis, MO). Microdialysis probes with 10 mm length, 0.5 mm diameter, and 100 kDa cut-off Polyethersulfone (PES) membranes were purchased from Harvard Apparatus (Holliston, MA). The 1.0 mL gastight syringe and syringe pump were purchased from BASi (West Lafayette, IN) and the MAB 20 peristaltic microdialysis pump from Watson Marlow Alitea (Stockholm, Sweden). The Nanodrop 2000 Spectrophotometer was purchased from Thermo Fischer Scientific Inc. (Waltham, MA).

B. Solutions

All solutions were prepared using 10 mM Phosphate Buffer Solution (PBS) with pH 7.4. Solutions of 6% Dextran 500, 100 μM MO, 100 μM FITC-4, and 100 μM FITC-40 in 10 mM PBS, respectively, were prepared as standards.

C. Push-Pull Microdialysis

The microdialysis pump and syringe were assembled as shown in Figure 4. A 0.5 mL microcentrifuge tube was filled with sample and placed on top of the stir plate. The microdialysis pump was perfused with 6% Dextran 500 in 10 mM PBS through the inlet tubing at 1 μL/min flow rate. Triplicate samples were collected using the microdialysis pump pushing at 1 μL/min with the MAB 20 peristaltic pump disconnected (0 pull). The MAB 20 was then connected and set to pull at flow rates 1, 2, 3, 4, 5, and 8 μL/min, respectively, with the microdialysis pump pushing at a constant 1 μL/min.

For each trial, a 0.2 mL collection vial was used to collect the dialysate. Each vial was weighed so the total volume collected could be determined. Prior to collection at each flow rate, the microdialysis pump and the MAB 20 were run for 20 min. to allow the system to equilibrate. This experiment was conducted at 22 °C and 1 atm pressure.

D. Vacuum Ultrafiltration

The microdialysis pump, syringe, and MAB 20 peristaltic pump were assembled, as shown in Figure 7, and perfused with 10 mM PBS at 5 μL/min for 20 min. to ensure no air was present in the lines. A 0.5 mL microcentrifuge tube was filled with sample and placed on top of the stir plate. The MAB 20 was disconnected

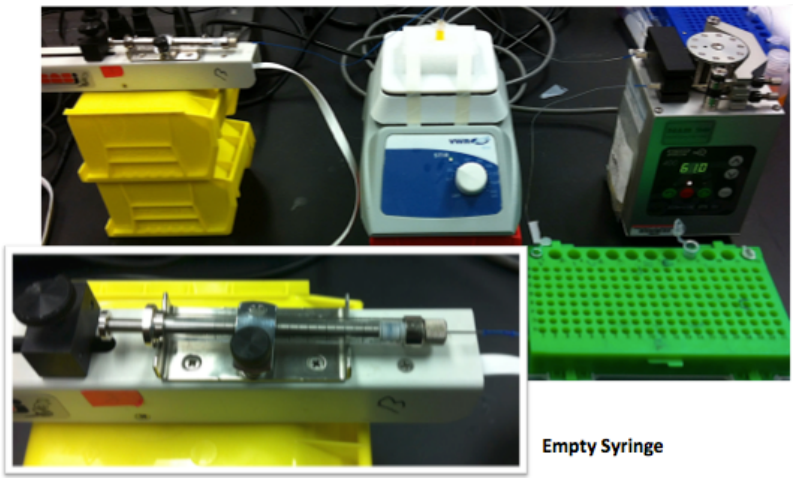

Figure 7. Vacuum Ultrafiltration experimental setup with empty syringe to seal off inlet tubing.

from the probe outlet tubing and triplicate samples were collected using the microdialysis pump pushing at 1 µL/min (0 pull). The syringe was then detached, emptied, and reattached to seal off the inlet tubing. The MAB 20 was reconnected to the outlet tubing and set to pull at 1, 2, 3, 4, 5, and 8 µL/min, respectively, with the microdialysis pump in the OFF position.

For each trial, a 0.2 mL collection vial was used to collect the dialysate. Each vial was weighed so the total volume collected could be determined. Prior to collection at each flow rate, the MAB 20 was run for 20 min. to allow the system to equilibrate. This experiment was conducted at 22 °C and 1 atm pressure.

E. Sample Measurement

Each analyte vial was weighed before and after sample collection to determine volume recovery. A calibration curve was constructed by measuring the absorbance, using the Nanodrop, of serial dilutions made from the 100 µM standards. The absorbance of the collected sample was then measured and referenced to the calibration curve to determine concentration recovery.

IV. Results and Discussion

A. Push-Pull Microdialysis

The percent recovery of MO, FITC-4, and FITC-40 using push-pull microdialysis are shown in Figure 8. Push-pull percent recovery for MO at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 70.16 ± 17.04, 67.80 ± 8.20, 65.44 ± 1.37, 69.84 ± 1.58, 72.71 ± 2.34, 74.51 ± 2.77, and 82.18 ± 0.25, respectively. Percent recovery of FITC-4 at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 35.17 ± 2.08, 26.17 ± 4.71, 48.50 ± 3.58, 67.83 ± 2.50, 73.83 ± 2.50, 76.50 ± 2.08, and 82.17 ± 4.02, respectively. FITC-40 percent recovery at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 2.99 ± 1.04, 3.38 ± 1.04, 8.45 ± 0.55, 28.62 ± 0.73, 32.41 ± 1.69, 35.64 ± 1.39, and 40.06 ± 0.59, respectively. MO percent recovery showed no significant variation from 0 to 8 µL/min pull flow rates using the difference of means statistical analysis ($p<0.05$, $n=3$). FITC-4 recovery decreased from 0 to 1 µL/min pull flow rates before increasing with higher pull flow rates thereafter. One potential explanation for this occurrence is the negative pressure exerted by the MAB 20 being equal to the positive pushing pressure causing a continual flow of perfusion fluid and decreasing resistance and residence time in the

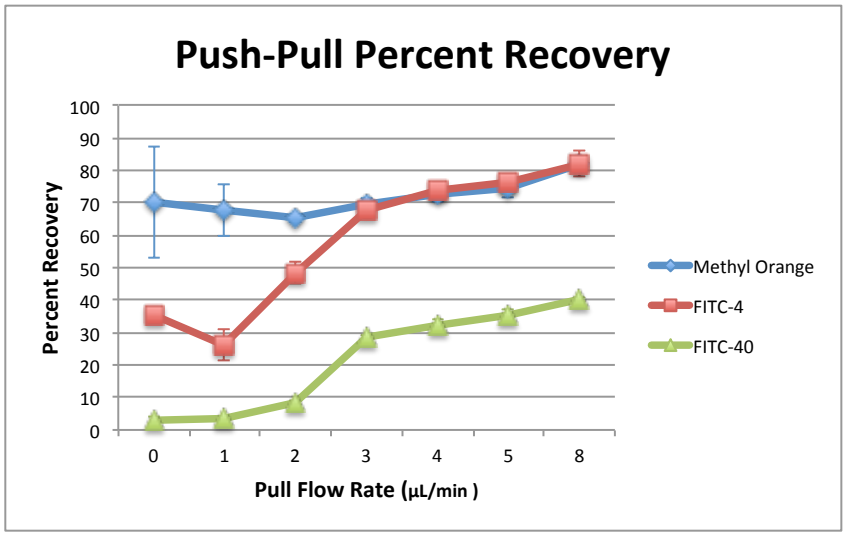

Figure 8. Push-Pull Percent Recovery.

probe for equilibration to occur with the analyte. Further testing is required to determine the cause or if this was an anomaly. FITC-40 recovery showed a dramatic increase from 2 to 3 µL/min pull ($p<0.05$, $n=3$) then steadily increased thereafter.

The volume recovery of MO, FITC-4, and FITC-40 using push-pull microdialysis are shown in Figure 9. Push-pull volume recovery for MO at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 1.16 ± 0.03, 1.02 ± 0.01, 1.99 ± 0.01, 2.96 ± 0.01, 3.12 ± 1.37, 4.81 ± 0.01, and 7.53 ± 0.05 µL/min, respectively.

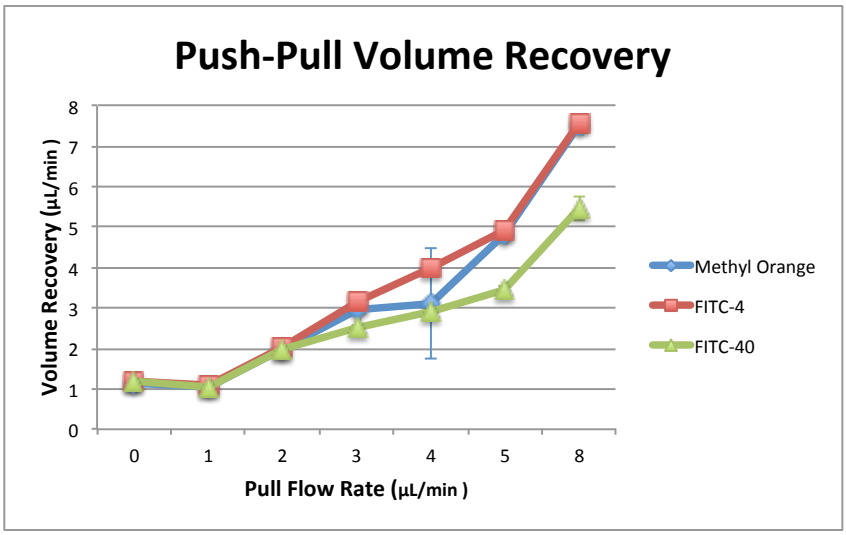

Figure 9. Push-Pull Volume Recovery.

Recovery of FITC-4 at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 1.19 ± 0.02, 1.07 ± 0.06, 2.04 ± 0.06, 3.14 ± 0.10, 4.01 ± 0.04, 4.94 ± 0.04, and 7.57 ± 0.21 µL/min, respectively. Recovery of FITC-40 at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 1.20 ± 0.03, 1.03 ± 0.02, 1.97 ± 0.04, 2.54 ± 0.05, 2.89 ± 0.09, 3.43 ± 0.10, and 5.46 ± 0.29 µL/min, respectively. Volume recovery of all three analyte using 1 µL/min push with 0, 1, and 2 µL/min pull, respectively, showed no significant variation ($p<0.05$, $n=3$). The recovery of MO and FITC-4 had similar volume recoveries at all pull flow rates with FITC-40 volume recovery being significantly less than MO and FITC-4 at 5 and 8 µL/min pull ($p<0.05$, $n=3$). The reason for this may be concentration polarization where there is a build

up of FITC-40 at the outside of the membrane. The hydrodynamic radius of FITC-40 vs. FITC-4 is 4.5 nm vs. 1.4 nm, respectively, while the inner lumen of the probe has approximately 9 nm pores limiting the rate of diffusion.

B. Vacuum Ultrafiltration

Figure 10 shows the percent recovery of MO, FITC-4, and FITC-40 using vacuum ultrafiltration. Vacuum ultrafiltration percent recovery for MO at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 64.24 ± 5.89, 91.97 ± 2.66, 93.44 ± 2.50, 91.71 ± 1.67, 93.57 ± 3.45, 97.31 ± 5.64, and 94.51 ± 3.45, respectively. Percent recovery of FITC-4 at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 33.17 ± 6.53, 90.50 ± 4.15, 96.17 ± 3.58, 95.17 ± 2.08, 95.83 ± 5.01, 95.17 ± 2.65, and 97.17 ± 2.65, respectively. FITC-40 percent recovery of at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 4.07 ± 0.33, 48.76 ± 15.08, 80.69 ± 9.23, 85.84 ± 4.97, 89.87 ± 6.39, 88.33 ± 3.59, and 91.48 ± 8.01, respectively. At 0 µL/min pull (using 1 µL/min push only), MO showed the highest recovery and FITC-40 the lowest, demonstrating recovery using conventional microdialysis decreases as analyte molecular weight increases. MO and FITC-4 showed similar recoveries at 1, 2, 3, 4, 5,and 8 µL/min pull, respectively.

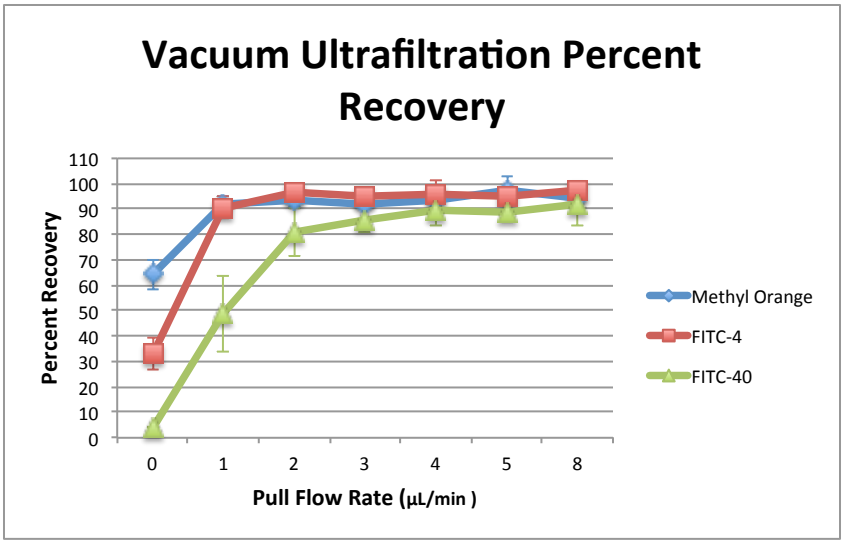

Figure 10. Vacuum Ultrafiltration Percent Recovery.

FITC-40 showed a significant increase from 0 to 2 µL/min pull ($p<0.05$, $n=3$), respectively, then leveled off thereafter.

The volume recovery of MO, FITC-4, and FITC-40 using vacuum ultrafiltration are shown in Figure 11. Vacuum ultrafiltration volume recovery for MO at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 0.88 ± 0.01, 1.04 ± 0.01, 2.04 ± 0.01, 3.01 ± 0.01, 3.88 ± 0.11, 4.85 ± 0.09, and 6.06 ± 1.48 µL/min, respectively. Recovery of FITC-4 at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 1.18 ± 0.02, 1.03 ± 0.00, 2.05 ± 0.02, 3.02 ± 0.03, 3.96 ± 0.02, 4.93 ± 0.01, and 7.55 ± 0.03 µL/min, respectively. Recovery of FITC-40 at 0, 1, 2, 3, 4, 5, and 8 µL/min pull was 1.15 ± 0.04, 0.93 ± 0.06, 1.68 ± 0.18, 2.22 ± 0.35, 2.60 ± 0.46, 2.58 ± 0.81, and 2.95 ± 0.85 µL/min, respectively. Volume recovery of all three analytes using 0, 1, and 2 µL/min pull, respectively, showed no significant variation ($p<0.05$, $n=3$). MO and FITC-4 continued to have similar recoveries at higher pull flow rates. FITC-40 recovery using 3, 4, 5, and 8 µL/min pull, respectively, was significantly less than MO and FITC-4 at the same flow rates ($p<0.05$, $n=3$).

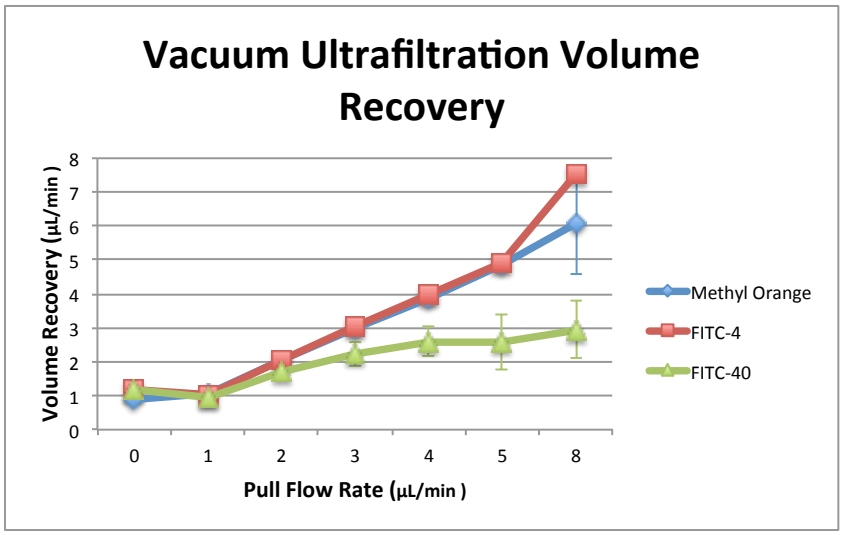

Figure 11. Vacuum Ultrafiltration Volume Recovery.

Overall, the vacuum ultrafiltration sample percent recoveries were higher than push-pull microdialysis percent recoveries. This is mostly due to the push-pull samples being diluted by the constant 1 μL/min push of perfusion fluid. Volume recoveries of MO and FITC-4 were similar using push-pull vs. vacuum ultrafiltration. However, FITC-40 volume recoveries were higher using push-pull rather than vacuum ultrafiltration due to the added 1 μL/min pushed perfusion fluid being collected in addition to the analyte sample.

V. Conclusion

Vacuum ultrafiltration sampling provided greater percent recovery while push-pull microdialysis yielded higher volume recovery. When using vacuum ultrafiltration, adequate analyte fluid is needed since it pulls from and quickly depletes sample volume. Because vacuum ultrafiltration uses no perfusion fluid, it is best suited for *in vitro* sampling while push-pull microdialysis would be best suited for *in vivo* sampling studies.

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