# MICROFLUIDIC PURIFICATION OF EXTRACELLULAR VESICLES FROM RAW BLOOD SAMPLES

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## ABSTRACT

We introduce a novel use of porous polymer monoliths as nanoporous membranes to purify extracellular vesicles in microfluidic chips. Using in-situ UV-initiated photopolymerization, membrane elements can be patterned in the desired region in microchannels by simple contact mask lithography with reasonable resolution. Pore size can be manipulated by changing the ratio of porogenic solvent to prepolymer solution, and was tuned to a size proper for the filtration of vesicles (~100 nm). Employing the membrane as a filter with a driving force of either pressure bias or electrophoretic injection, vesicles were extracted from the blood of melanoma grown mice and their contained RNA was analyzed.

## KEYWORDS

filtration, porous polymer monolith, exosome, microvesicle, electrophoresis.

## INTRODUCTION

Various mammalian cells, including tumor cells, actively secrete extracellular vesicles (EVs), also known as exosomes and microvesicles, into their surrounding environments, eventually leading to their existence in many bodily fluids. Their cargo of proteins, lipids, and nucleic acids representative of their origin cells are being recognized as increasingly important as they may be used as disease indicators that can be gathered noninvasively [1]. For analysis based on vesicles, EVs from the sample fluid must be isolated from coexisting material, e.g. cells and debris. Currently used techniques to accomplish this include serial centrifugation and ultra-centrifugation, which is laborious, or immobilization based on a characteristically expressed surface molecule, which may be overly specific. Compared to existing protocols, microfluidic filtration is rapid, nonexclusive and well suited to processing small sample volumes.

We report the development of a purification system based on size exclusion filtration or electrophoretic separation and demonstrate its effectiveness by purifying vesicles from the whole blood of healthy and melanoma grown mice. The proposed device can be fabricated simply in PMMA substrates and the membrane is formed by photopolymerization of a porous polymer monolith (PPM) [2]. EVs were filtered using the membrane and we found greater amounts of vesicle contained RNA in filtered samples compared to conventional ultracentrifugation techniques.

## IN-SITU PHOTOPATTERNING OF MEMBRANE ELEMENTS

A crossflow microchip is fabricated in a PMMA substrate by CNC micromilling. The membrane is then synthesized in the region defined by a mask by filling the channels with a prepolymer solution and exposing the chip to UV light, as shown in Figure 1a and b. The prepolymer solution consists of a monomer, glycidyl methacrylate (GMA), cross-linker, ethylene glycol dimethacrylate (EGDMA), photo-initiator, 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) and porogenic solvent, methanol. GMA was chosen for its negative surface charge, which prevents similarly charged vesicles from adhering to its surface by electrostatic attraction. Additionally, the chemical similarity in the acrylate groups of the PMMA substrate and monomer GMA allowed the monolith to remain firmly anchored to the channel walls after synthesis, without an accompanying surface modification (Figure 1c and d) [3].

## MEMBRANE POROSITY CHARACTERIZATION

The porosity characteristics of the filter were tested by passing various mixtures of calibrated particles through the channels until an upper and lower bound were found for each tested recipe of PPM. Therefore, estimated pore size was found as a range between the rejected and permeated species of particles, as in Figure 2a and b. We found that the proportion of porogenic solvent was the most direct parameter to control the pore size characteristics. Gradually decreasing the porogen content led to the formation of tight, closely packed networks of monolith beads and effectively decreased the pore size, as compared between Figures 2c and 2d.

## EXTRACTION OF EXTRACELLULAR VESICLES

We devised two schemes for carrying out microfluidic isolation of EVs from blood samples. Using the membrane as a size exclusion filter, we separated vesicles from cells and large debris by injecting whole blood under pressure through the microfluidic device. EVs and small proteins could pass through the membrane unimpeded while cells and other components larger than the minimum pore size of ~300 nm were unable to permeate. The heterogeneity of PPM pores lends itself well to filtering particles of high size contrast since large components are blocked by the larger features of meso and macro-pores while small material continues to pass unrestricted through the smallest pores of the network. Up to 5 µl of filtrate could be extracted from raw blood samples before the filter reached its clogging limit. The membrane with embedded support posts can withstand high pressures (typically 1.5 bar) with



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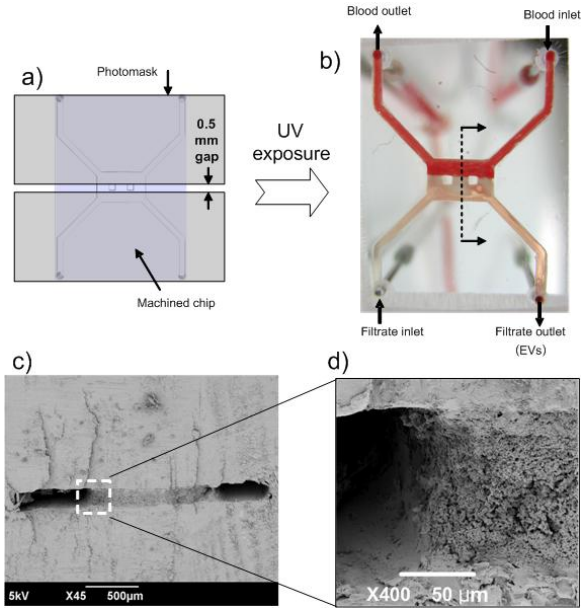


**Figure 1.** The proposed microfluidic device. (a) A cross channel microfluidic chip is filled with prepolymer solution and exposed to UV light through a mask. (b) After photopolymerization, the synthesized monolith acts as a semi permeable barrier. (c) SEM image of the section cut shown in "b". (d) High magnification of the region in "c" shows the monolith structure is strongly integrated into the channel walls and that sharp resolution between the monolith and the channel lumen is possible.

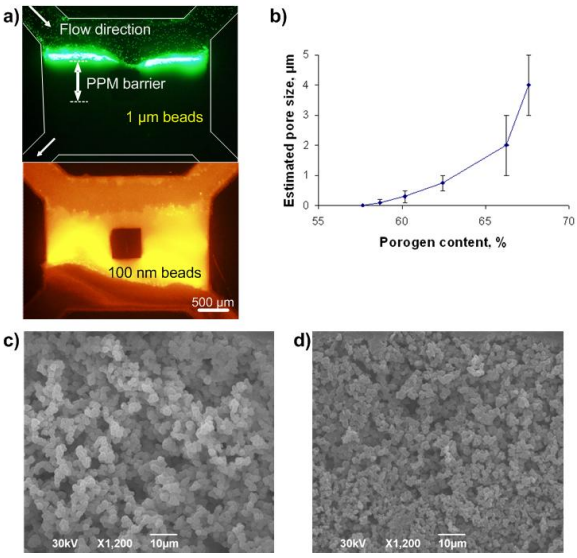


**Figure 2.** Porosity characterization demonstrated by permeation of different sized fluorescent beads. (a) Fluorescent micrographs showing the separation of 1 µm (green) and 100 nm (red) polystyrene beads. Arrows indicate the flow direction. (b) Porosity can be controlled by adjusting the proportion of porogenic solvent. (c) SEM image of a section cut of a 67% monolith formed in a channel; a relatively open structure is formed. (d) At 58% a tighter network is formed. A recipe of 60% porogen was used for vesicle filtration, corresponding to a pore size of ~300 nm.

failure occurring first in plastic tubing attachment points first rather than in the chip.

In a parallel technique, electrophoretic injection was employed as an alternative driving force to propel particles across the filter and increase the separation efficiency of vesicles from proteins. When bias voltage is applied across the sample reservoirs, particles possessing a surface charge are moved through the membrane, but large cells remained blocked. Exploiting the electrophoretic mobility difference between cell-derived vesicles and soluble proteins, the preferable migration of vesicles was realized. This allows EVs to permeate and accumulate on one side of the membrane while less mobile proteins are carried away by the cross flow stream before they are able to penetrate, thereby increasing the purity of EVs in the filtrate with respect to unit protein content.

EVs purified by both filtration methods were verified to retain their intact morphology by TEM observation (Figure 3). Also, the membranes presented here can be washed and reused multiple times while maintaining their original properties.

## ANALYSIS OF VESICLE RNA

The half-life of free RNA present in the blood stream is very short due to circulating ribonuclease, so the existence of non degraded RNA in the lysates of collected samples must be mostly due to the existence of RNA containing vesicles or cells. Compared to raw whole blood, cell free pressure filtered samples were rich in RNA, demonstrating an enrichment of EVs from the available inlet blood. RNA from EVs collected from a conventional ultracentrifugation protocol was also compared and we found the proposed microfluidic pressure filtration system could gather EVs more efficiently from smaller starting volumes. The electrophoretic injection mode could gather EVs at a rate similar to the ultracentrifuge procedure (Figure 4). In the instance of pressure filtration we found there to be more RNA in a given volume of blood in subjects with tumors compared to healthy controls ($p$ value $< 0.05$), supporting the notion that tumor cells are very active in releasing RNA containing EVs.

Furthermore, the electrophoretic mode was able to exclude distracting proteins due to its more selective separation mechanism. In the case of tumor grown mice, twice as much RNA could be extracted per unit protein content (38.9 ±10.1 ng/100µg protein) in the case of electrophoretic filtration compared to pressure filtration and ultracentrifugation (12.7 ±3.4 ng/100µg and 18.8 ±5.4 ng/100µg respectively).

## CONCLUSIONS AND FUTURE WORK

The successful implementation of mass transport control via semipermeable membranes installed in microfluidic chips allows a new level of flexibility in integrating on-line sample processing steps. We introduced a simple microfluidic filtration system to rapidly extract EVs from small samples, allowing them to be analyzed downstream. EVs are growing in importance as research tools and have especially high potential for the diagnosis and early

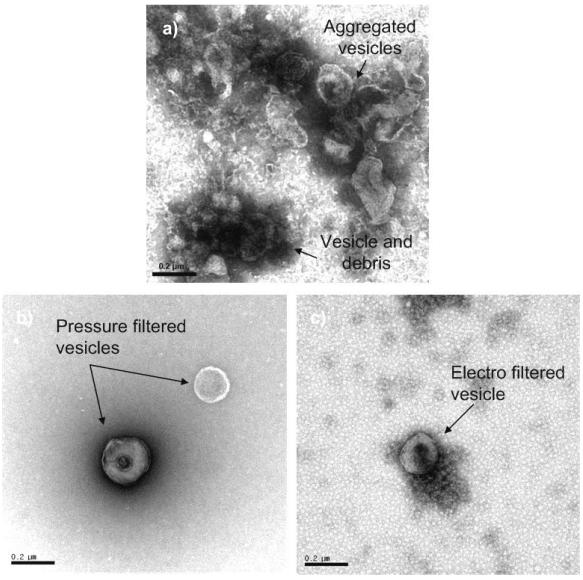


**Figure 3.** TEM images of tumor subject mouse blood vesicles purified by (a) conventional ultra-centrifugation, (b) pressure driven microfluidic filtration, and (c) electrophoretic-injection driven filtration. The native morphology of vesicles is preserved in the microfluidic filtration methods, while ultracentrifugation causes many vesicles to fuse together and aggregate with proteins. Filtered EVs are round and show a characteristic cup shape.

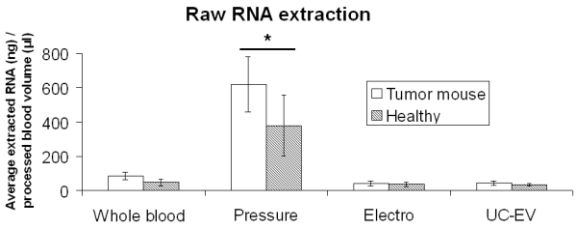


**Figure 4.** Total RNA extracted from whole blood and vesicle samples purified by various filtration methods. UC-EV denotes ultracentrifuge isolated EVs. Pressure filtered samples were found enriched in RNA containing EVs compared to whole blood and showed even greater recovery efficiency than existing ultracentrifuge procedures. Also, a difference in EVs was found between tumor and healthy subjects in the case of pressure samples. *, $p < 0.05$.

monitoring of cancer. In future work, we aim to employ microfluidicly isolated blood-borne EVs in elucidating the status of tumors.

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