Chapter 29

An Integrated Double-Filtration Microfluidic Device for Detection of Extracellular Vesicles from Urine for Bladder Cancer Diagnosis

Li-Guo Liang, Ye-Feng Sheng, Sherry Zhou, Fatih Inci, Lanjuan Li, Utkan Demirci, and ShuQi Wang

Abstract

Extracellular vesicles (EVs) are present in a variety of bodily fluids and they play an important role in cellular communications and signal transduction mechanisms. Studies have shown that the number of EVs and EV-associated biomarkers (i.e., proteins, nucleic acids and lipids) can be used to aid clinical diagnosis. Although ultracentrifugation is commonly used for EV isolation, it is not practical for clinical settings. Here, we developed an integrated double-filtration device that isolated and enriched EVs from urine, and subsequently detected/quantified EVs from urine via microchip ELISA. Results showed that the concentration of EVs was significantly elevated compared to healthy controls. Receiver operating characteristic analysis demonstrated that this integrated EV quantification device had a sensitivity of 81.3% at a specificity of 90% (16 bladder cancer patients and eight healthy controls). Thus, this integrated device shows great potential to supplement urine cytology for diagnosis of bladder cancer in point-of-care (POC) settings.

Key words Filtration, Extracellular vesicles (EVs), Microchip ELISA, Bladder cancer, Diagnostics

1 Introduction

Bladder cancer is the top two malignancies of the genitourinary system, with an annual incidence of 20.1 per 100,000 men and women in the US [1]. The 5-year survival rate of in situ and localized bladder cancer is approximately 77.5%, whereas it drops to 34.5–5.2% for regional and distant bladder cancer [2]. Since the majority of bladder cancer patients (~80–90%) experience painless hematuria, or additionally with urination discomfort, bladder cancer is often underdiagnosed, potentially leading to development of muscle-invasive tumor. Thus, high-risk populations should be routinely screened for bladder cancer to avoid serious clinical outcomes such as radical cystoprostatectomy. However, cytology, cystoscopy and other diagnostic approaches such as BTA stat and BTA TRAK

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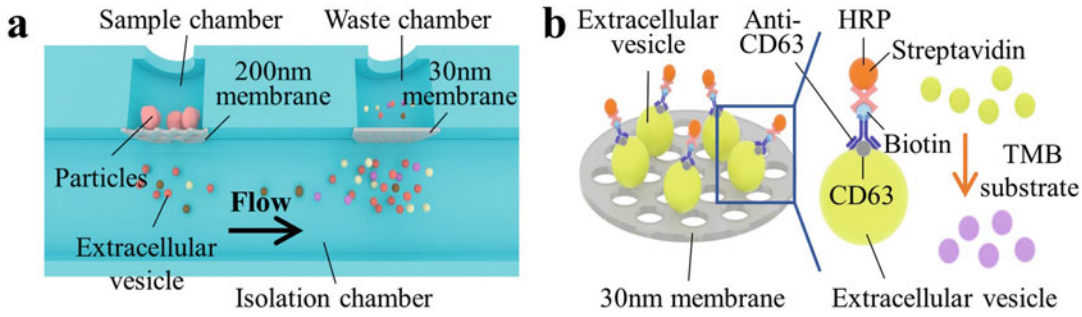

Fig. 1 Working principle of an integrated EV detection device. (**a**) Illustration of the double-filtration process for isolation and enrichment of EVs from urine. (**b**) Illustration of microchip-ELISA for detection/quantification of EVs

tests, ImmunoCyt and UroVysion cannot meet the requirement for early diagnosis or screening of bladder cancer [3, 4].

EVs are microvesicles derived from cells with a diameter ranging approximately 40–100 nm [5]. Studies have shown that EVs play an important role in cellular communication, especially for cancer metastasis [6, 7], and that the number of EVs and EV-associated biomarkers such as proteins, nucleic acids and lipids can be used to aid diagnosis of cancer [8, 9]. Although ultracentrifugation can be used to isolate EVs from biological samples, the requirement for a prohibitive ultracentrifuge and its time-consuming process constitute a major bottle for clinical translation of EV-related diagnostics [10–12]. Here, we present an integrated double-filtration device that can isolate, enrich, and quantify EVs from urine via microchip ELISA for differentiating bladder cancer patients from healthy controls (Fig. 1). This double-filtration microfluidic device eliminates the need for an expensive, bulky ultracentrifuge for EV-related research and potentially clinical diagnosis in a less-equipped laboratory. Since simple, rapid and inexpensive diagnostics are preferably needed for resource-limited settings [13, 14], this integrated EV diagnostic platform holds great potential for POC testing.

2 Materials

All solutions are prepared and stored as recommended by manufacturers.

2.1 Cell Culture

1. Roswell Park Memorial Institute (RPMI) 1640 basal media.
2. 10% heat-inactivated fetal bovine serum (FBS).
3. 100 U/mL penicillin.
4. 100 μg/mL streptomycin.

5. Hanks' Balanced Salt Solution, With Ca^{2+} and Mg^{2+}(137.93 mM NaCl, 5.33 mM KCl, 4.17 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.493 mM $MgCl_2$, 0.407 mM $MgSO_4$, 0.441 mM KH_2PO_4, 0.338 mM Na_2HPO_4 and 5.56 mM D-glucose).
6. Trypsin–EDTA Solution (0.25% trypsin, 0.02% EDTA and 0.02% Phenol Red in 0.01 M phosphate buffer saline, PBS).
7. PBS, pH 7.0 (Sangon Biotech, Shanghai, China).

2.2 Urine Samples

1. Urine samples are collected from bladder cancer patients ($n = 16$) and healthy donors ($n = 8$).

2.3 Devices

1. Poly(methyl methacrylate) (PMMA) (3M Company, St. Paul, MN).
2. Double-sided adhesive (DSA).
3. Nuclepore track-etched membrane (GE Healthcare Life Science, Shanghai, China).
4. Laser cutter (VSL 2.30, Universal Laser System Inc., Scottsdale, AZ).
5. Micropump (TS-2A, Longer, Baoding, China).
6. Epoxy adhesive (DP 460NS, 3M, Shanghai, China).

2.4 ELISA

1. Biotinylated anti-CD63 (1 mg/mL) (Abcam, Cambridge, MA).
2. Streptavidin-labeled horseradish peroxidase (HRP) (1 mg/mL) (Abcam, Cambridge, MA).
3. Alexa Fluor 488-labeled anti-CD9 (1 mg/mL) (Abcam, Cambridge, MA).
4. 100 and 500 nm fluorescent particles (Ocean Nanotech, Springdale, CA).
5. Tubing Φ 0.8 mm × 1.85 mm (Longer, Baoding, China).
6. Tetramethyl benzidine (TMB) Chromogenic Reagent (Sangon Biotech, Shanghai, China).
7. 0.22 μm filtering device (Sangon Biotech, Shanghai, China).
8. PBS (pH 7.0) (Sangon Biotech, Shanghai, China).
9. BCA kit (Sangon Biotech, Shanghai, China).
10. Nuclease-free H_2O (Sangon Biotech, Shanghai, China).
11. Fluorescence microscope (Leica DM4000, German).
12. Scanning electron microscope (SEM) (SU-8010, Hitachi, Tokyo, Japan).
13. 96-well ELISA plate (Coring, Shanghai, China).
14. Spectrophotometer (Molecular Devices, San Francesco, CA).

3 Methods

3.1 Device Fabrication

1. Four layers of PMMA with a thickness of 2, 1, 1 and 2 mm, are excised to dimensions of 20 × 40 × 6 mm (Fig. 2).
2. Two circular openings with a diameter of 1.8 mm are excised on the on the first PMMA (2 mm in thickness) layer to form an inlet and outlet (*see* **Note 1**).
3. Two circular openings with a diameter of 10 mm are excised on the second PMMA layer (1 mm in thickness) to form a sample chamber and waste chamber.
4. The third PMMA layer (1 mm in thickness) had two openings with a diameter of 10 mm, which are connected through a microchannel with a width of 1.5 mm.
5. The fourth PMMA layer (2 mm in thickness), which had no openings, is used to form the base of the device.
6. Two Whatman membranes with a pore size of 200 and 30 nm in diameter are included between the second and third PMMA layer.
7. Four layers of DSA (50 μm in thickness) with corresponding configurations are excised using a laser cutter to assemble the double-filtration device using a non-photolithography method [15] (*see* **Note 2**).
8. The formed double-filtration device had two circular chambers (78.5 μL) above the membranes and two circular chambers (78.5 μL) below (*see* **Note 3**).

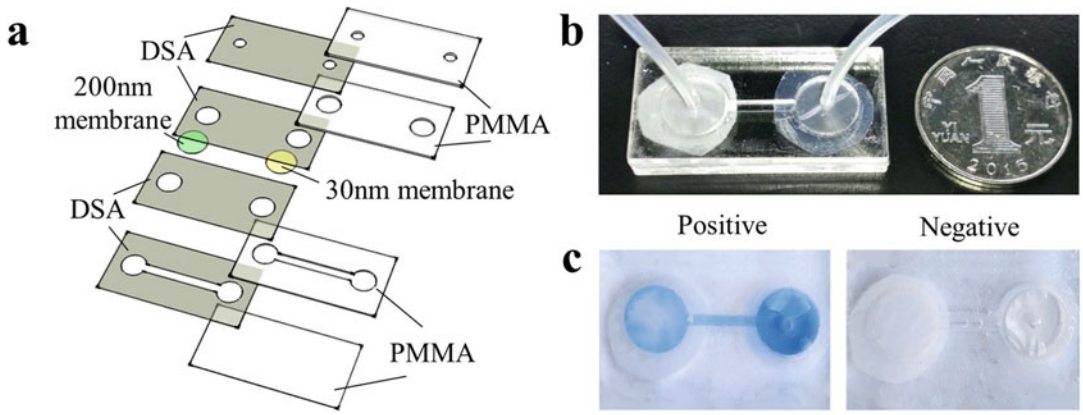

Fig. 2 Assemble of an integrated EV detection device. (**a**) Layer-by-layer structure of the device. (**b**) Actual image of the device. (**c**) Images of the device after EV detection using microchip ELISA

3.2 Cell Culture

1. Human bladder cancer cell line T24 is cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humid atmosphere with 5% CO_2 (*see* **Note 4**).
2. T24 cells are harvested on day 3, and the number of cells is counted using a hemocytometer.
3. The supernatant is collected after centrifuging 100 mL of T24 cell culture with a cell density of 10^6 cells/mL at room temperature for 15 min at 2000 × *g* (*see* **Note 5**).

3.3 EV Isolation Using Ultracentrifugation

3.3.1 EVs Isolation from Cell Medium

1. 100 mL of cell culture media with a cell density of 10^6 cells/mL is harvested after T24 cell culture for 4 days.
2. 100 mL of cell culture media is centrifuged at 2000 × *g* for 15 min.
3. To pellet EVs, the supernatant is centrifuged at 100,000 × *g* at 4 °C for 70 min in an SW32 Ti rotor.
4. The supernatant is discarded carefully.
5. 200 μL of PBS is used to rinse the pellets gently and then discarded with caution.
6. Crude EV-containing pellets are suspended in 1 mL of PBS.
7. Isolated EVs are kept at −80 °C for storage until use.

3.3.2 EVs Isolation from Urine

1. 10 mL of urine is centrifuged at 2000 × *g* at room temperature for 15 min to remove cells (*see* **Note 5**).
2. The supernatant is carefully removed.
3. The supernatant is centrifuged at 100,000 × *g* at 4 °C for 70 min in an SW32 Ti rotor.
4. Isolated EVs are kept at −80 °C for storage until use.

3.4 EV Isolation Using Double Filtration

1. Urine samples or T24 culture are centrifuged at 2000 × *g* for 15 min to remove cells and cell debris (*see* **Note 5**).
2. 8 mL of urine sample or cell medium is continuously injected into a double-filtration device at a flow rate of 40 μL/min (Fig. 3a).
3. 400 μL of PBS is injected into the device at a flow rate of 40 μL/min for three times.

3.5 Microplate ELISA

1. EVs isolated from T24 cell cultures by ultracentrifugation are serially diluted to 10^6, 3.3×10^5, 1.1×10^5, 3.7×10^4 and 1.23×10^4 AU.
2. 100 μL of EV suspension is added to each well of a 96-well ELISA plate and incubated 4 °C for 2 h.
3. 100 of μL BSA (1%) is added to the 96-well ELISA plate and incubated for 1 h at 37 °C (*see* **Note 6**).

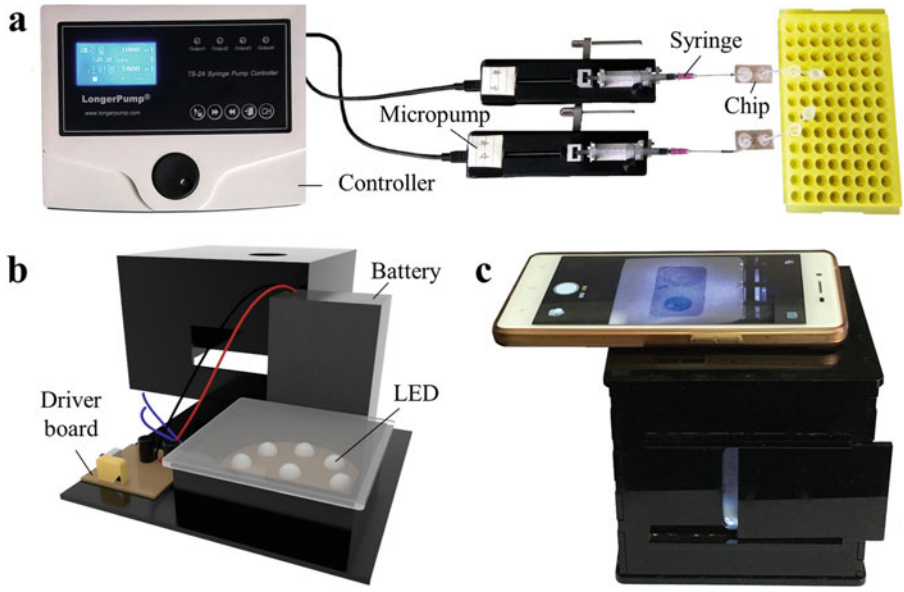

Fig. 3 Experimental setup for isolation, enrichment, and quantification of EVs. (**a**) Image of device operation with the aid of a micropump. Samples and reagents were flowed into the double-filtration device at a flow rate of 40 μL/min. (**b**) Schematic of the detection setup for cell phone imaging. The battery-operated imaging setup was illuminated using an LED. (**c**) Actual image of the cell phone-based detection setup

4. 200 μL of PBS (pH 7.0) is used to wash the plate for three times.
5. 100 μL of biotinylated anti-CD63 antibody (1:200) is added to the plate and incubated at 37 °C for 1 h (*see* **Note 6**).
6. The plate is washed with 200 μL PBS per well (pH 7.0) for three times.
7. 100 μL of streptavidin-labeled HRP (1:2000, 100 μL per well) is added and incubated for 1 h at 37 °C (*see* **Note 6**).
8. The plate is washed using 200 μL of PBS for three times.
9. 100 μL of TMB is added into each well and incubated at 37 °C in darkness (*see* **Note 6**).
10. 50 μL of stopping solution is then added to each well to terminate color development.
11. The color intensity of each well is measured using a spectrophotometer at a wavelength of 450 nm.

3.6 Microchip ELISA

1. EVs are isolated from urine using the double-filtration device as described in Subheading 3.4.
2. 300 of μL of biotinylated anti-CD63 antibody (1:200) is injected into the device at a flow rate of 40 μL/min and then incubated at 25 °C for 1 h (Fig. 3a).

3. 300 μL of PBS is injected three times to the device at a flow rate of 40 μL/min.
4. 500 μL of air is injected once to completely remove PBS (*see* **Note 7**).
5. 300 μL of streptavidin-labeled HRP (1:2000) is injected to the device at a flow rate of 40 μL/min and then incubated at 37 °C for 1 h (*see* **Note 8**).
6. The device is washed using PBS and air as **steps 3** and **4** as described above.
7. 300 μL of TMB is injected into the device and then incubated at 37 °C for 10 min in darkness (*see* **Notes 8–10**).
8. Color development is imaged using a smartphone (OPPO R7, Guangdong, China) in an in-house imaging box (Fig. 3b and c) (*see* **Notes 11** and **12**).
9. Cellphone images are then transferred to a laptop for image analysis using ImageJ.
10. R channel values of blue color is extracted and used to plot the standard curve at the function of EV concentrations, as previously reported [16, 17].

4 Notes

1. The power of the laser cutter used for cutting PMMA is 75% at a speed of 10%.
2. The power of the laser cutter used for cutting DSA is 10% at a speed of 10%.
3. The surroundings of the double-filtration device are sealed using Epoxy adhesive to avoid leakage or evaporation.
4. A commercial 0.22 μm filter device is used to remove potential bacterial contamination in culture media.
5. Urine samples and cell culture are centrifuged at a speed of 2000 × *g* for 15 minutes to remove cells and cell debris.
6. The 96-well plate is covered using Parafilm.
7. Air wash is performed following PBS washing.
8. Devices are placed in a wet box during incubation steps at 37 °C wherever applied to avoid evaporation.
9. Foil is used to cover the double-filtration device during the injection and incubation of streptavidin-labeled HRP and TMB.

10. TMB solution is warmed up to room temperature before use.
11. The double-filtration device with developed blue color is imaged immediately after 10-min incubation of TMB to avoid signal saturation.
12. Blue color developed in double-filtration devices is imaged in a homemade black box with predefined lightening conditions to avoid the interference with ambient lights. EVs isolated from T24 cell culture by ultracentrifugation.

In this study, we present an integrated device that can isolate, enrich, and quantify EV from urine samples without referring to ultracentrifugation. Based on size-exclusion, this device isolated EVs with a size ranging from 30–200 nm, and meanwhile enriched EVs in a defined small volume (165 μL) from a starting volume of 8 mL. In addition, we developed a 96-well plate ELISA for detection of EV using anti-CD63 labeling (Fig. 4a). This ELISA detection strategy was further miniaturized into the double-filtration device for on-chip detection using a cellphone (Fig. 4b) [16, 17]. This integrated EV quantification platform was validated using 16 urine samples from bladder cancer patients and eight urine samples from healthy controls. Box-whisker analysis clearly shows that the concentration of EVs was significantly elevated compared to healthy controls (Fig. 4c), indicating that the concentration of urine EVs can be potentially used as a diagnostic biomarker to differentiate bladder cancer patients from healthy controls. Receiver operating characteristic analysis demonstrates that this integrated EV quantification device had a sensitivity of 81.3%, at a specificity of 90% (Fig. 4d). This integrated EV microchip offers an inexpensive option for translating EV-related research or clinical diagnosis for less-equipped laboratories. Nevertheless, clinical validation of this integrated device in a large population would be helpful to assess the diagnostic value of urine EV as a biomarker for detection of bladder cancer or other urogenital cancers.

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Competing financial interests: Dr. U. Demirci is a founder of, and has an equity interest in: (1) DxNow Inc., a company that is developing microfluidic and imaging technologies for point-of-

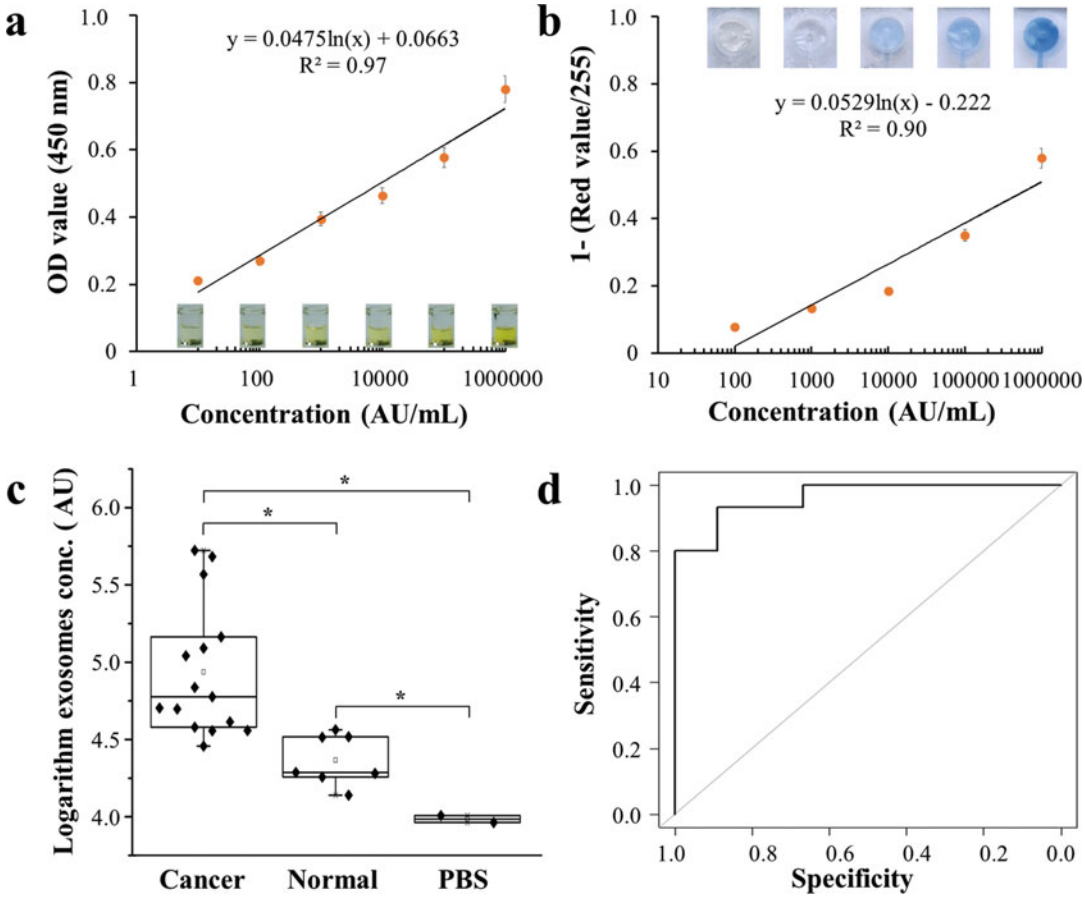

Fig. 4 Quantification of EVs using 96-well plate and microchip ELISA. (**a**) Standard curve of 96-well plate ELISA for detection of EVs. EVs were isolated from T24 cell culture using ultracentrifugation, and the lowest detection point was defined 10 artificial unit (AU). (**b**) Standard curve of microchip ELISA for detection of EVs. EVs were isolated from T24 cell culture using an integrated double-filtration device. Images were taken using a cell phone and R channel values of *blue color* were extracted using ImageJ and then plotted at the function of EV concentrations. (**c**) Box-whisker analysis of log-transformed EV concentrations of 24 urine samples (bladder cancer = 16, healthy controls = 8). The concentration of urinary EVs from bladder cancer was significantly higher that from healthy controls ($p < 0.05$). (**d**) Receiver operating characteristic (ROC) analysis of microchip ELISA for differentiating bladder cancer patients from healthy controls, and the microchip ELISA had a sensitivity of 81.3% at the specificity set to 90.0%

care diagnostic solutions, and (2) Koek Biotech, a company that is developing microfluidic IVF technologies for clinical solutions. U.D.'s interests were viewed and managed in accordance with the conflict of interest policies.

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