



Graphene oxide-cellulose composite cryogels for extracellular vesicle isolation

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ABSTRACT

Herein, we present an innovative approach for the selective and high-efficiency isolation of extracellular vesicles (EVs) derived from microfluidic chips that replicate the tumor microenvironment. This is achieved through the development of graphene oxide-cellulose-based composite cryogels imprinted with EVs. In the experiments, MCF-7 cells were cultured under flow conditions within microfluidic chips, simulating the dynamic tumor microenvironment. The isolated EVs were comprehensively characterized in terms of biological, morphological, and quantitative attributes using scanning electron microscopy and nanoparticle tracking analysis. Subsequently, the EVs were imprinted onto graphene oxide-cellulose-based composite cryogels, yielding a novel polymeric material designed for highly selective EV isolation. The physicochemical properties of the composite cryogels were thoroughly analyzed using multiple characterization techniques, and their kinetic performance was evaluated under varying parameters (pH, concentration, temperature, ionic strength, flow rate and selectivity). The maximum adsorption capacity was calculated to be 231 particles/g in a pH 5.0 buffer solution at room temperature. Non-composite and non-imprinted composite cryogels were also prepared for comparison experiments. Finally, the efficacy and validation of the platform were confirmed via high-performance liquid chromatography (HPLC) analysis, demonstrating its potential as a powerful tool for EV isolation in cancer research.

1. Introduction

Extracellular vesicles (EVs) are tiny, membrane-bound vesicles (50–150 nm in diameter) that encapsulate diverse functional biomolecules, including nucleic acids, proteins, and lipids [1]. These vesicles are released by numerous cell types, both healthy and diseased, and serve as critical mediators of intercellular communication [2]. By carrying bioactive components including proteins, lipids, nucleic acids, and signaling molecules, EVs regulate various physiological and pathological processes [3,4]. Their pivotal role in cellular crosstalk has garnered considerable interest in biomedical research, particularly for their potential applications as biomarkers for disease diagnostics, drug delivery vectors, and reservoirs of therapeutic molecules [5]. Despite these promising attributes, achieving highly specific and efficient EV isolation remains a fundamental challenge in the field, necessitating the

development of advanced and reproducible isolation techniques [6]. Breast cancer is a global health concern, affecting women of all ages after puberty, with incidence rates increasing with age. The MCF-7 cell, a widely studied human breast cancer model, was originally derived in 1970 from a pleural expression of a 69-year-old woman with metastatic breast cancer [7]. The name “MCF-7” originates from the Michigan Cancer Foundation-7, where these cells were first isolated [8,9]. MCF-7 cell-derived EVs have been extensively investigated for their role in breast cancer biology and progression [10]. MCF-7 cells serve as a crucial model for understanding hormone-responsive breast cancer [11]. The EVs secreted by these cells are key players in tumor progression, intercellular communication, and drug resistance, highlighting their significance in cancer research and potential therapeutic strategies [12].

Cryogels, characterized by their highly porous three-dimensional

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architecture, offer significant potential for addressing critical biomedical and biotechnological challenges. These materials are synthesized via cryogelation, a process in which gelation occurs at subzero temperatures, leading to the formation of an interconnected porous network [13]. This unique fabrication method enables precise control over pore size and morphology, making cryogels highly versatile for applications including adsorption, drug delivery, separation, and tissue engineering [14,15]. Their inherent high surface area and porosity, coupled with a mechanically stable yet permeable structure, enhance their functionality across various disciplines [16].

Graphene oxide (GO), a chemically functionalized derivative of graphene, consists of a single layer of carbon atoms with oxygen-containing groups [17]. These groups enhance GO's hydrophilicity, dispersibility, and reactivity, making it ideal for composite materials [18,19]. Cellulose, the most abundant natural polymer, is biodegradable, biocompatible, and renewable, but lacks advanced functional properties like electrical conductivity [20]. Combining GO with cellulose forms a hybrid material with enhanced mechanical strength, electrical conductivity, and chemical stability [21,22]. This composite holds great promise for diverse applications in environmental remediation, energy storage, and biomedical engineering [23–25]. A graphene oxide-cellulose-based cryogel is a highly porous material synthesized by gelation of GO-cellulose composites under subzero conditions, forming interconnected pores that facilitate efficient mass transport and adsorption [26,27]. GO enhances mechanical strength, electrical conductivity, and molecular interactions, while cellulose ensures biocompatibility and structural integrity [28]. The high porosity of cryogels enables effective adsorption and separation of molecules or ions, with GO further improving mechanical, electrical, and surface properties [29].

In this study, we developed a novel graphene oxide-cellulose-based composite cryogel (MIP-GO) for selective EVs isolation from microfluidic chips that mimic the cancer microenvironment. EVs derived from MCF-7 cells were biologically, morphologically, and numerically characterized using scanning electron microscopy (SEM) and nanoparticle tracking analysis (NTA) before being imprinted onto the cryogel to create a targeted isolation platform. The MIP-GO cryogels were extensively characterized through SEM, thermogravimetric analysis (TGA), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), and swelling tests. To optimize EV isolation, various parameters—including pH, concentration, temperature, ionic strength, flow rate, and selectivity—were investigated. Non-imprinted and non-composite composite cryogels were also synthesized to compare the adsorption capacity of polymers. HPLC studies were operated to validate the experimental findings, confirming the efficiency of MIP-GO as a highly selective EV isolation platform.

2. Materials and methods

2.1. Preparation of EV sample

MCF-7 cells were initially cultured in microfluidic chips under continuous flow conditions for 4 days. The medium collected in the syringe pump after the culture process contained EVs. In this study, an ultrafiltration-based strategy was applied for EV isolation. Two filter membranes with pore sizes of 200 nm and 50 nm were used. Initially, the cell culture medium was directed to the 200 nm filter via tubing, where larger particles were retained on the filter surface, while nanoparticles smaller than 200 nm passed through. These nanoparticles were then transported via a microchannel to the second filter membrane with a 50 nm pore size. EVs were subsequently collected from the region containing the 50 nm filter membrane using tubing. The entire flow was measured using a syringe pump operating at a constant flow rate (40 $\mu\text{L}/\text{min}$).

2.2. Characterization of EVs

Nanoparticle Tracking Analysis (NTA, NS300, Malvern, UK) was used to determine EV concentration and size distribution based on Brownian motion using a 488 nm laser. Measurement parameters were: capture = 5, duration = 60 s, temperature = 25 °C, viscosity = 0.92 mPa·s. Western blotting was performed to verify EV purity and markers. Proteins were separated by SDS-PAGE (10 % resolving, 4 % stacking), and transferred onto PVDF membranes. After blocking with 5 % milk-TBST, membranes were incubated overnight at 4 °C with rabbit anti-CD63 (EV marker) and rabbit anti-Calnexin (negative control) primary antibodies, followed by HRP-conjugated secondary antibody. Bands were visualized using Luminol/Enhancer solution and imaged on a Bio-Rad ChemiDoc MP system.

2.3. Preparation of graphene oxide-cellulose composite

Graphene oxide (GO) was synthesized from synthetic graphite flakes (<20 μm) using an improved version of Marciano's method [30]. In brief, concentrated H_2SO_4 (360 mL) and H_3PO_4 (40 mL) were added to a mixture of graphite flakes (3.0 g, 1 wt%) and KMnO_4 (18.0 g, 6 wt%). The reaction mixture was maintained at 50 °C with continuous stirring for 12 h. After cooling to room temperature, it was poured into an ice bath, followed by the slow addition of 3 mL H_2O_2 . The product was purified via dialysis for 48 h. The resulting suspension was filtered using a 0.1 μm membrane filter and subsequently dried at room temperature to yield GO powder for composite preparation.

Approximately 4.5 g of GO powder was then dispersed in 90 mL of ultrapure water and sonicated for 15 min. The dispersion was cooled to 0 °C in a refrigerator before adding 6.0 g of NaOH and 4.0 g of urea, followed by 4.0 g of microcrystalline cellulose. The mixture was gently stirred in an ice bath for 10 min, then incubated at 20 °C for 2 h, and further stirred at room temperature for 30 min. The resulting blend was extruded through a 1 mL syringe into 2 M HNO_3 , where coagulation occurred overnight. The composites were thoroughly rinsed with tap water for 30 min, followed by distilled water for 15 min. The obtained GO-cellulose composite was stored in distilled water at room temperature, with the GO content determined to be 4.15 wt%.

2.4. Preparation of composite cryogels

A 0.71 g of acrylamide (AA) monomer and 500 μL of EV template molecule (1×10^8 particles/mL) mixed with 1 mL of pure water and interacted with each other for 30 min 20 rpm to obtain pre-complex. Then, 1 mL of GO-cellulose were added to this mixture. 1.3 mL of 2-hydroxyethyl methacrylate (HEMA) comonomer dissolved in 1.2 mL of pure water and 283 mg of *N,N'*-methylene bisacrylamide (MBA) cross-linker dissolved in 10 mL pure water, separately. Finally, 20 mg of potassium persulfate (KPS) and 25 μL of *N,N,N',N'*-tetramethylethylenediamine (TEMED) were used as an initiator pair. The final mixture was poured in syringes and frozen at −18 °C to form the composite cryogel. After 24 h polymerization, the composite cryogel was thawed at room temperature and washed extensively with water to remove impurities, ensuring it was ready for next experiments. Scheme 1 shows the step-wise workflow and chemical reaction flow of the composite cryogel.

2.5. Characterization analyses

The characterization analyses of isolated EVs were accomplished by using SEM (QUANTA 400F), and NTA (NS300, Malvern). Following the composite cryogel preparation, the characterization analysis of composite cryogels were also obtained by using SEM, TGA (TA Instrument), XPS (K-Alpha, ThermoFisher Scientific), FTIR (Thermo Fisher Scientific, Nicolet iS10), and swelling test for physical and chemical content of composite cryogels. The spectrum was acquired between 400 cm^{-1} and

Step-wise Workflow

Monomer (AA)
Co-monomer (HEMA)
Cross-linker (MBA)
Solvent: Water

Initiators (KPS, TEMED)
GO Cellulose

Extracellular Vesicle (EV) Addition

Composite Polymer Mixture

Filling the Syringe Tubes/Columns

Polymerization at Cryostat System (-18°C for 24 hours)

Initiation

Potassium Persulfate + TEMED → Radical formation → TEMED Radical + Bisulfate Radical

Bisulfate Radical → Sulfate Radical anion + H⁺

Sulfate Radical anion + Acrylamide → Acrylamide Radical

Propagation

Acrylamide Radical + HEMA → Polymer chain with radical end

Polymer chain with radical end + HEMA → Propagated polymer chain

MBA Crosslink & Termination

Polymer chain with radical end + MBA → Crosslinked polymer network

Cryogel Structure

The cryogel is composed of GO (Graphene Oxide) and Cellulose. It is used for EV Capturing through Hydrogen Bonding. After EV Elution, the cryogel structure is shown as a hollow cylinder.

Scheme 1. Step-wise workflow and chemical reaction flow of composite cryogel.

4000 cm^{-1} wavenumbers. The superficial topology of composite cryogel was examined by SEM analysis. The thermal stability of the composite cryogel was evaluated using TGA at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ under a nitrogen atmosphere. The elemental composition of the composite cryogel was examined by XPS. For analysis, dried cryogel samples were mounted on the sample holder using copper tape and evacuated for 30 min. After acquiring the general elemental survey spectrum, the analysis was completed. To determine the equilibrium swelling degree, dried composite cryogels were first weighed (W_0) and then immersed in 10 mL of pure water. The swollen cryogels were removed at regular time intervals (0–30 min), gently blotted to remove surface water, weighed (W_{sw}), and the swelling degree was calculated using the following equation:

$$\text{Swelling degree} = \frac{W_0 - W_{\text{sw}}}{W_0} \times 100\%$$

2.6. Adsorption analyses

In the adsorption analyses, the performance of the composite cryogel was evaluated under varying conditions, including pH (4.0–8.0), EV concentration (1.0×10^7 – 5.0×10^8 particles/mL), temperature (4–40 $^{\circ}\text{C}$), ionic strength (0.05–1.0 M NaCl) and flow rate (5–20 rpm) to assess their effects on adsorption capacity. In adsorption experiments, 10 mL buffer solution was passed through a composite cryogel for 2 h. After adsorption, desorption process was carried out with 10 mL of 0.1 M NH_3 solution for 1 h. Subsequently, the composite cryogel was washed with water for 1 h to prepare next experiment. Following these procedures, the absorbance values of the samples were measured at 280 nm using ultraviolet-visible (UV-Vis) spectroscopy (Shimadzu, Model 1601). All experiments were performed in triplicate to ensure reproducibility. The adsorbed amount (Q) was calculated with the following equation:

$$Q = (C_i - C_f) \times \frac{V}{m}$$

Here, Q represents the amount of EVs adsorbed per unit mass of cryogel (particles/g), C_i and C_f denote the initial and equilibrium concentrations of EVs (particles/mL), V is the volume of the EV solution (mL), and m is the dried mass of the composite cryogel (g).

2.7. Selectivity analyses

In selectivity analyses, individual solution of MCF-7 EVs and human embryonic kidney cell (HEK-293) EVs were prepared at the same concentration of 1.0×10^8 particles/mL in 10 mL of pH 5.0 and the sample solutions were added to imprinted and non-imprinted composite cryogels, at 10 rpm for 2 h at 25 $^{\circ}\text{C}$.

After adsorption, the MCF-7 and HEK-293 EVs bound to the cryogel were desorbed using 0.1 M NH_3 solution for 1 h, followed by washing with pure water for an additional 1 h. Subsequently, the absorbance of each sample was measured at 280 nm using UV-Vis spectroscopy. All experiments were performed in triplicate to ensure reproducibility. The selectivity coefficients (k) for the adsorbed EVs, considering both the template and the competitor (MCF-7 and HEK-293), were calculated according to the following equation:

$$k = \frac{Q_{\text{template}}}{Q_{\text{competitor}}}$$

where Q_{template} represents the amount of adsorbed EVs (MCF-7), and $Q_{\text{competitor}}$ denotes the amount of adsorbed competitor EVs (HEK-293). The relative selectivity coefficient (k'), which accounts for the effect of imprinting on EV selectivity, can be determined using the following equation:

$$k' = \frac{k_{\text{imprinted}}}{k_{\text{non-imprinted}}}$$

where $k_{\text{imprinted}}$ denotes the selectivity coefficient of the imprinted composite cryogel carrier, and $k_{\text{non-imprinted}}$ represents the selectivity coefficient of the non-imprinted composite cryogel carrier.

2.8. Validation analyses

EV solutions were prepared to assess whether composite cryogels could be used for EV determination in a complex sample medium. EV solutions were prepared at six different concentrations for both imprinted and non-imprinted composite cryogels (1.0×10^7 – 5.0×10^8 particles/mL) to create the calibration curve. The detection of EVs in sample solutions was confirmed using an HPLC system in an isocratic elution mode (Dionex, Ultimate HPLC). The mobile phase used for elution was prepared using an acetonitrile solution (90:10, v/v ACN: H_2O). The samples, including EVs, were analyzed with a flow rate of 0.5 mL/min using a reverse-phase HPLC C18 column (Phenomenex, 5 μm). Also to verify the imprinting efficiency the HPLC analyses of EVs for imprinted and non-imprinted composite cryogels were evaluated for different EV concentrations (1.0×10^7 – 5.0×10^8 particles/mL).

3. Results and discussion

3.1. Characterization analyses

All characterization analyses, including SEM, NTA, FTIR, TGA, XPS, and swelling degree, were conducted to examine the topology, physical, and chemical properties of the isolated EVs and composite cryogels. SEM offers a rapid approach for characterizing the morphology and distribution of EVs. As one of several micro- and nanoscale vesicles released by cells, EVs can be distinguished by their morphology and size distribution. Since these particles are typically smaller than 300 nm and cannot be detected using optical methods, electron microscopy is essential for detailed morphological characterization [31]. SEM images revealed that the EVs, examined at different magnifications, exhibited a uniform, equidimensional, and round morphology (Fig. 1A). The ability to quickly detect and quantify microvesicles, including their size and concentration, in both cell cultures and clinically relevant body fluids, provides significant advantages. NTA results showed that the EVs isolated from cells cultured at a flow rate of 2 $\mu\text{L}/\text{min}$ had a 138.2 nm of average size and a 1.81×10^{10} particles/mL of concentration (Fig. 1B).

FTIR analysis of the composite cryogel revealed several absorption peaks: N–H at 3332 cm^{-1} , C–H at 2941 cm^{-1} , C=O at 1712 cm^{-1} and 1659 cm^{-1} (strong), N–O at 1557 cm^{-1} , C–H at 1448 cm^{-1} and 1389 cm^{-1} , C–O at 1240 cm^{-1} –1074 cm^{-1} (strong), C=C at 1023 cm^{-1} (strong), and 510 cm^{-1} (Fig. 2A). The presence of carboxyl and amino groups indicates the amphoteric nature of the polymer [32]. Absorption peaks from HEMA in the composite cryogel included a broad peak at 3332 cm^{-1} , strong peaks at 1712 cm^{-1} and 1659 cm^{-1} , and additional peaks at 1151 cm^{-1} and 1074 cm^{-1} [33]. Thermal stability of the composite cryogel was assessed by TGA (Fig. 2B). Mass loss up to 200 $^{\circ}\text{C}$ was attributed to the evaporation of trapped water and moisture, with a 4.9 % loss observed. Decomposition of organic groups began around 220 $^{\circ}\text{C}$ and continued to 420 $^{\circ}\text{C}$. Notably, 11.11 % of the composite cryogel's mass was retained after 800 $^{\circ}\text{C}$ [34]. XPS analysis provided insight into the elemental composition and chemical speciation of the cryogel surface (Fig. 2C). The wide-scan spectra showed C-C/C-H, C–O, and C=O bonds at binding energies of 284.71, 286.41, and 288.33 eV with the C–O bond at 531.79 eV. N1s scanning revealed an amide bond at 399.65 eV [35]. SEM analysis at various magnifications revealed that GO-cellulose was incorporated into the cryogel structure without hindering macropore formation (Fig. 2D). The cryogel exhibited super-macroporous structures, with pore sizes much larger than EVs size,

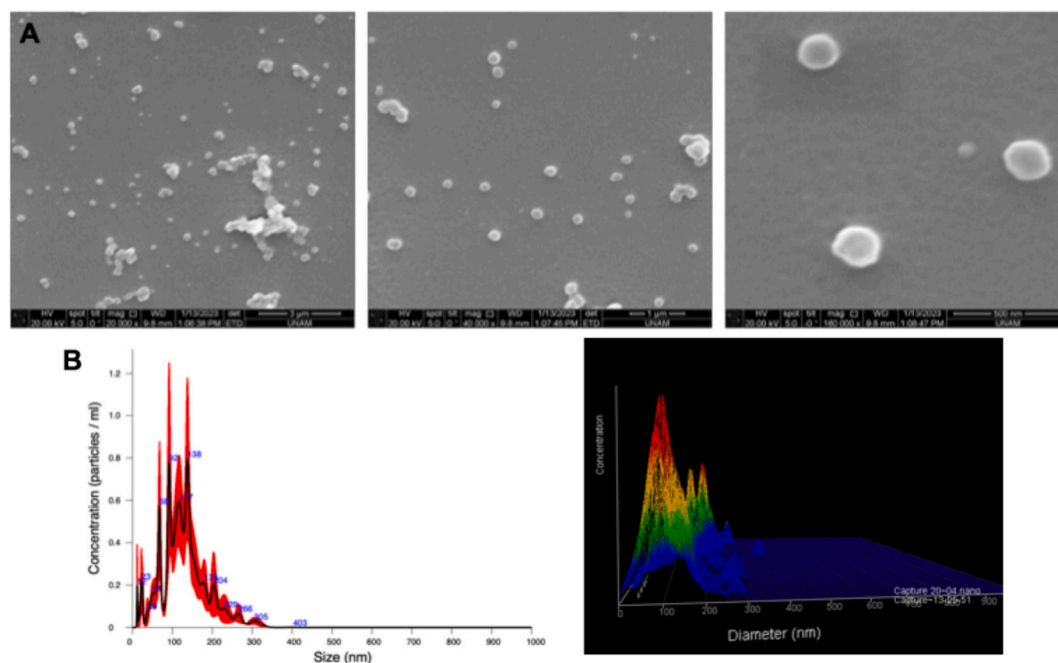


Fig. 1. SEM images (A) and NTA analysis (B) of isolated EVs.

allowing easy penetration. Channels with diameters of approximately 50–100 μm were observed in the cavities of dense polymeric walls.

Controlling the shape and size of super-macroporous cryogels is a combination of chemical formulation and physical processing during cryogelation. The overall shape of the cryogel is mainly dictated by the mold in which the polymerization occurs. The cryogel takes the exact shape of the mold (in our approach, it is a syringe). Using molds with grooves can impart specific surface features. In addition, the pore size is influenced by ice crystal formation during cryogelation, since ice acts as a porogen. Moreover, the orientation is pivotal to create aligned channels, enhancing flow and mass transfer. The swelling degree of the composite cryogel was calculated as 767 g $\text{H}_2\text{O}/\text{g}$ cryogel. Swelling occurred rapidly within 2 min, stabilizing after 10 min (Fig. S1).

3.2. Adsorption analyses

The optimal experimental parameters were established to evaluate the performance of the composite cryogel in calculating the amount of adsorbed EVs. To assess the effect of pH, adsorption studies were conducted using a series of buffers at pH values of 4.0, 5.0, 6.0, 7.4, and 8.0, with an EV concentration of 1.0×10^8 particles/mL at 25 $^\circ\text{C}$, under conditions of no ionic strength (I.S.) and a flow rate (F.R.) of 10 rpm. A calibration curve was obtained using an EV solution of known concentration to compare the concentration of the sample with the EVs in the solution. According to Fig. S2, EV samples with different concentrations absorb at 280 nm with approximately 99.83 % accuracy. The calculated absorbance capacity (Q) values in all experiments were obtained using the equation $y = 0.0024x + 0.0017$ obtained from the calibration curve. As Fig. 3A demonstrated the maximum adsorbed EVs value was obtained at pH 5.0. The hydrophilic monomer AA and the hydrophilic cross linker MBA were employed in the imprinting process to diminish non-specific adsorption and improve biocompatibility [36,37]. Furthermore, the use of acrylamide monomers facilitated the development of an artificial antibody with steric synergistic and electrostatic effects, enabling effective molecular imprinting of the composite polymer to target the EV membranes [37]. In an acidic environment, EV biomolecules concentrations increase significantly compared to a neutral environment. That is, an acidic pH may rise the stability of EVs in vitro, and as a result, the isolation can occur with a higher yield [38]. Adsorption analyses were

conducted at four different temperature values including 4, 25, 32 and 40 $^\circ\text{C}$. When the temperature effect was examined, it was concluded that the most appropriate temperature value was 25 $^\circ\text{C}$ with 1.0×10^8 particles/mL of EV sample at pH 5.0 without I.S. at 10 rpm R.S. (Fig. 3B). As the bindings between EVs and composite cryogel started to decrease as the temperature rise, the adsorbed EV value also decreased. The adsorption stayed an effect of electrostatic interactions [39]. Different ionic strength values (0.05–1.0 M NaCl) were used on EV adsorption with 1.0×10^8 particles/mL of EV sample at pH 5.0 at 25 $^\circ\text{C}$ at 10 rpm R.S. for testing ionic strength (NaCl) effect (Fig. 3C). When the effect of ionic strength was investigated, it was observed that increasing the salt concentration led to a reduction in EV adsorption, attributed to the weakening of interactions between the EVs and the composite cryogel. This phenomenon may be explained by NaCl molecules engaging in electrostatic interactions with the EVs, thereby blocking or competing with the available binding sites [40]. Four different flow rate values (5, 10, 15 and 20 rpm) were applied with 1.0×10^8 particles/mL of EV sample (I.S. = 0 M) at pH 5.0 at 25 $^\circ\text{C}$ for checking the effect of rate (Fig. 3D). When the effect of flow rate was examined, it was concluded that the most ideal flow rate was 10 rpm. The high flow rates should be avoided in order to minimize the loss of the binding capacity of composite cryogel [41].

EV samples with different concentrations (1.0×10^7 , 2.5×10^7 , 5.0×10^7 , 1.0×10^8 , 2.5×10^8 and 5.0×10^8 particles/mL) were interacted with both imprinted, non-imprinted composite and also non-composite cryogels at pH 5.0 (I.S. = 0 M) and 25 $^\circ\text{C}$ at 10 rpm. The adsorbed EV value increased with increased EV concentration up to 1.0×10^8 particles/mL and then it started to decrease (Fig. 4A). This may be because of the binding sites begin to fill after this concentration. Moreover, the results indicated that the imprinted composite cryogel possessed superior EV adsorption capability (231 particles/g vs 48 particles/g) instead of non-imprinted composite cryogel because of the non-specific interactions and binding sites precisely tailored to accommodate EVs [39]. The maximum adsorbed EV amount was also obtained as 136 particles/g for non-composite cryogel (Fig. 4B). It was concluded that graphene oxide-cellulose increased the composite cryogel in EV adsorption performance [42]. The relationship between the enrichment efficiency and the pore size is in the heart of how cryogel structure controls performance in applications. In most systems, enrichment efficiency first

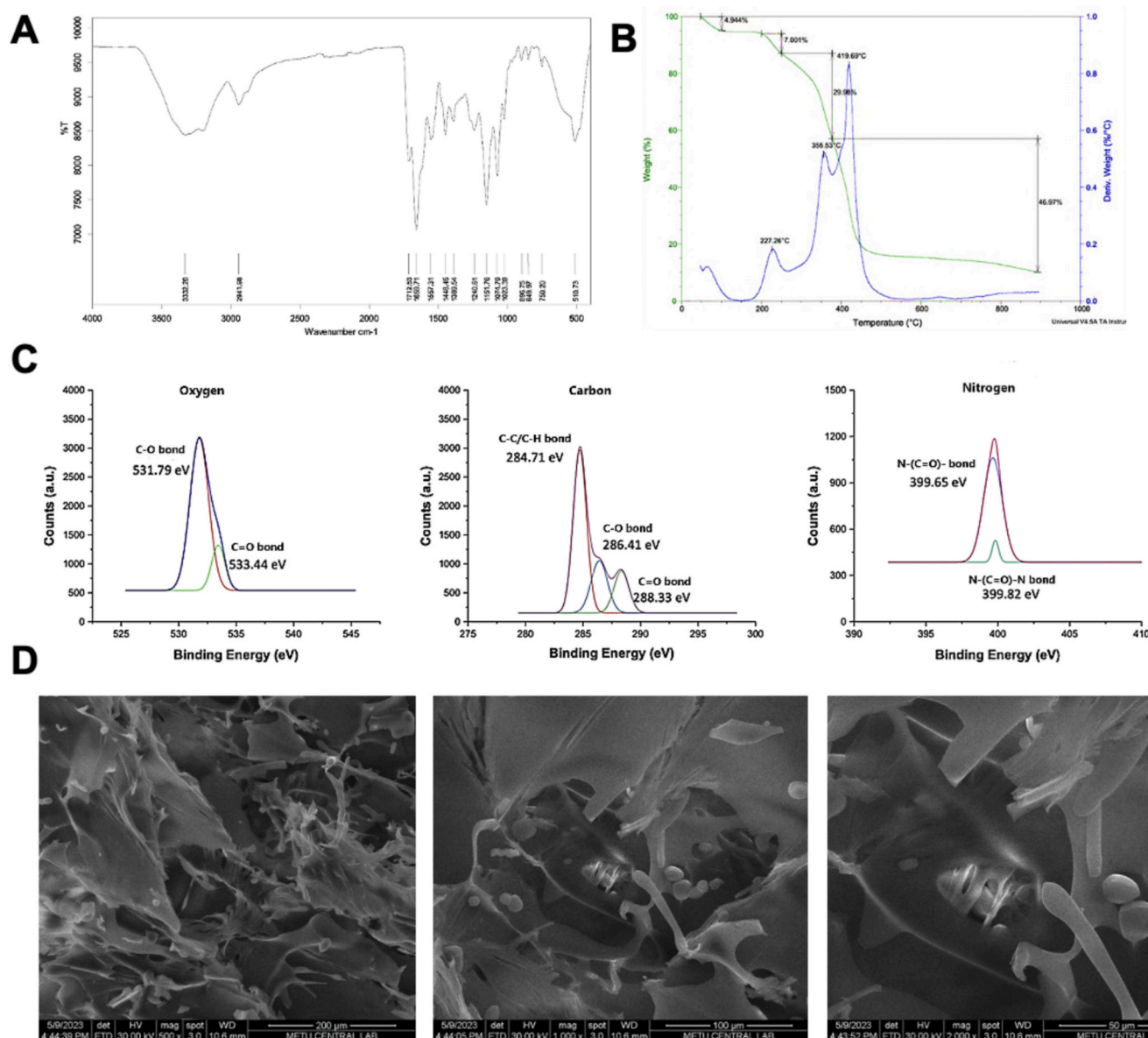


Fig. 2. FTIR (A), TGA (B), XPS (C) and SEM (D) analysis of composite cryogels.

increases with pore size (better flow and accessibility), then plateaus or decreases as pores become too large (loss of surface area and binding sites). Moreover, EVs can be efficiently eluted from the cryogel materials. After capture, the EVs were released using a mild elution procedure that preserves their structural integrity. Regarding the recovery, we systematically evaluated the efficiency of our method across a range of EV concentrations. The recovery percentage (%) was shown to assess the accuracy and reliability of the method and calculated as the ratio of the desorbed (found) and residual EVs to the initially applied (added) amount, ranging from approximately 83.3 % at the lowest concentration to slightly above 117.9 % at higher concentrations (Table S1). These results demonstrate that our approach provides robust and reproducible EV recovery, with minor variations at low concentrations likely due to measurement sensitivity. Overall, the data confirm that the method allows effective EV isolation and release while maintaining vesicle integrity.

3.3. Selectivity analysis

Selectivity analysis is crucial for assessing the performance of

molecularly imprinted polymers (MIPs), as it measures their capacity to discriminate target molecules from structurally similar analogs. Because the synthetic recognition sites in MIPs are specifically designed to match the size, shape, and functional groups of the template molecule, demonstrating high selectivity is fundamental to ensuring binding specificity [43]. In the selectivity analysis, the comparison of MCF-7 and HEK-293 EVs was evaluated. The results indicate that the cavities formed in the imprinted composite cryogel exhibited specific recognition for MCF-7 EVs, suggesting that these cavities were better matched in size and shape to MCF-7 EVs compared to HEK-293 EVs. Although both types possess similar functional groups the observed selectivity suggests that subtle differences in surface protein composition, membrane rigidity, or glycosylation patterns between MCF-7 and HEK-293 EVs may have contributed to the differential binding behavior (Fig. S3) [44]. Such findings highlight that molecular imprinting was governed not only by chemical similarity but also by subtle topological and structural features, resulting in relatively low non-specific interactions with non-target EVs. When compared to imprinted and non-imprinted composite cryogels, the adsorbed EVs amount (Q) decreased from 231 particles/mL to 48 particles/mL for MCF-7 EV adsorption

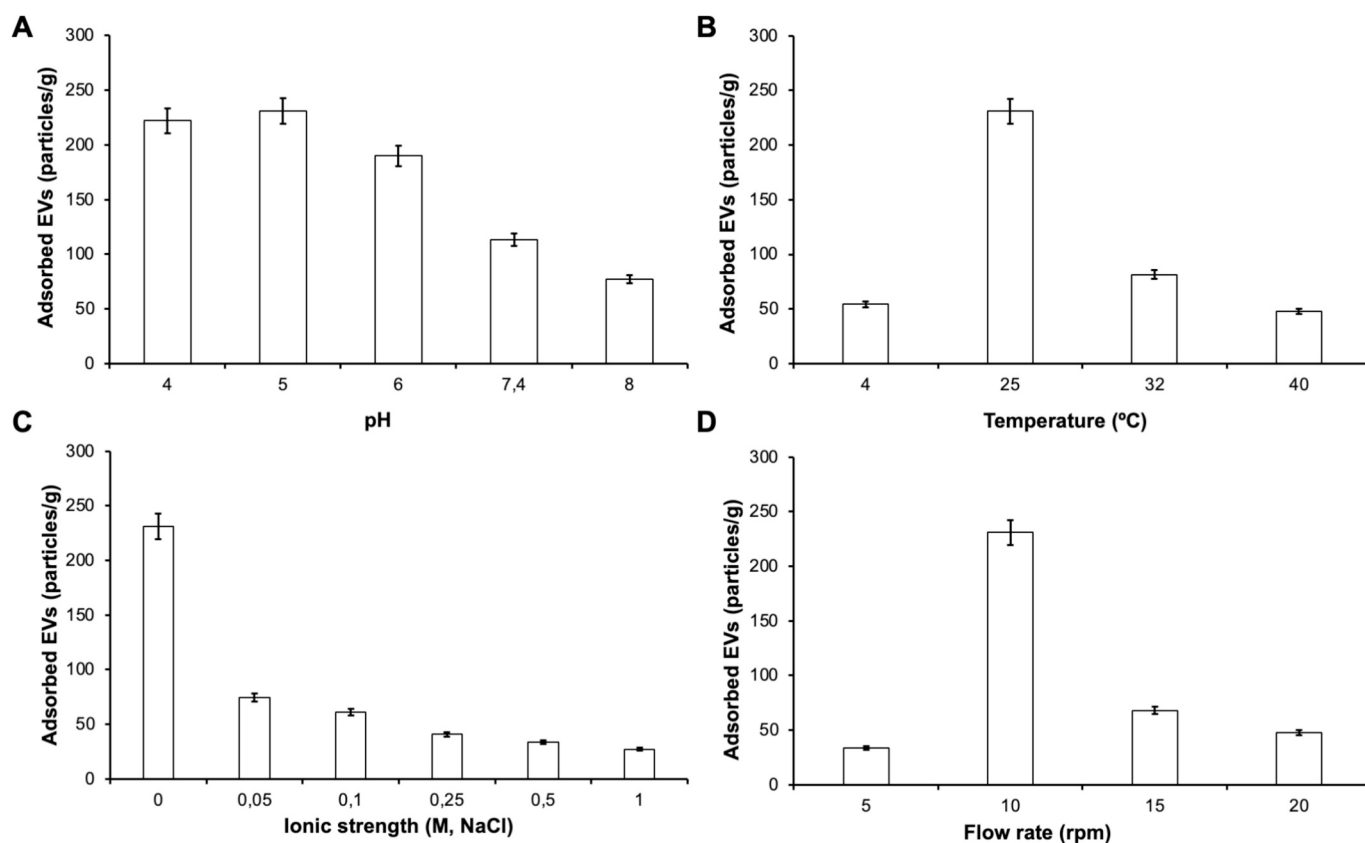


Fig. 3. Effect of pH (A), temperature (B), ionic strength (C) and flow rate (D) on the adsorption of EVs.

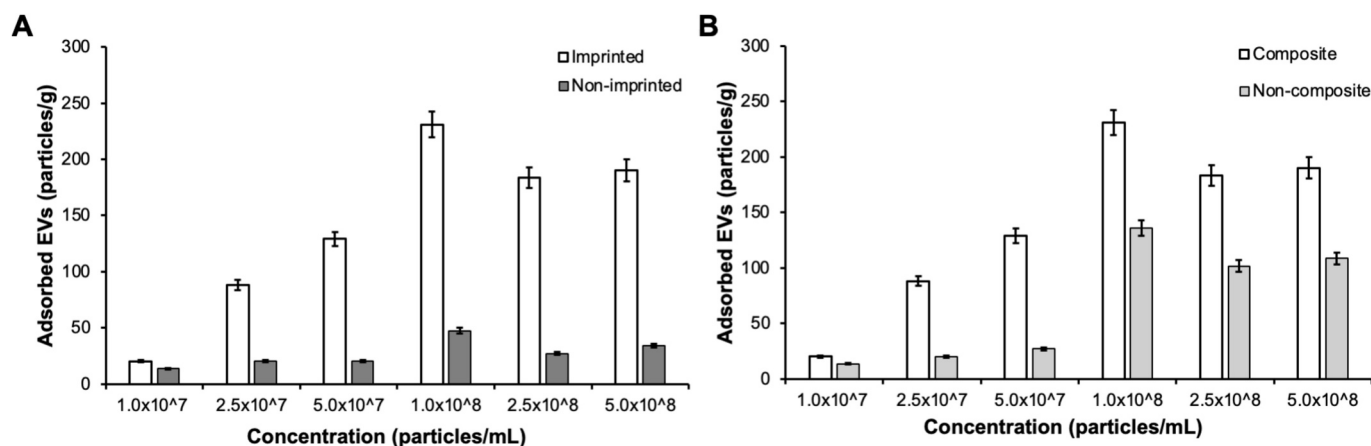


Fig. 4. Effect of concentration on the adsorption of EVs for imprinted and non-imprinted composite (A) and composite and non-composite (B) cryogels.

(Table S2). Mathematical calculations yielded a relative selectivity coefficient (k') of 3.04, indicating that the imprinted composite crygel exhibited a significantly higher affinity for adsorbing MCF-7 EVs compared to the non-imprinted composite crygel. In addition to this quantitative evidence, it is important to note that several EV subtypes and biomolecular complexes — such as microvesicles (100–1000 nm), apoptotic bodies (500–2000 nm), and lipoprotein complexes (10–100 nm) — overlap with EVs (50–150 nm) in size. However, the imprinted GO–cellulose composite cryogels operate through molecular-level recognition rather than mere size exclusion. During the imprinting process, MCF-7-derived EVs were used as templates to create recognition cavities complementary in both geometry and surface chemistry. These sites interact specifically with characteristic exosomal membrane

components (CD9, CD63, CD81, TSG101, Alix), enabling selective enrichment of bona fide EVs over other vesicle types. While microvesicles and apoptotic bodies may display partial overlap in physical dimensions, their distinct protein composition, lipid organization, and membrane topology markedly reduce their affinity for the imprinted sites. Lipoprotein complexes, lacking exosomal surface proteins, show negligible binding. This specificity was validated by the selectivity assays comparing MCF-7 and HEK-293 EVs, where a relative selectivity coefficient ($k' = 3.04$) and chromatographic resolution ($R_s > 1$) confirmed strong molecular discrimination.

3.4. Validation analyses

Selective adsorption of EVs using the imprinted composite cryogel was confirmed using the HPLC system. For this purpose, different EV solutions in the concentration range of 1.0×10^7 – 5.0×10^8 particles/mL were prepared and applied to the HPLC system to create a calibration curve (Fig. 5A). The adsorbed amount of EVs using the imprinted composite cryogel was validated by the HPLC system with a high regression coefficient ($R^2 = 0.9949$) and the equation ($y = 10.677x + 65.285$). In addition, the chromatograms of imprinted (Fig. 5B) and non-imprinted (Fig. 5C) composite cryogels showed that the differences between the initial (1-black) and final (2-blue) amount of adsorbed EVs. The area differences of imprinted and non-imprinted composite cryogels confirm that the imprinting process was performed successfully.

The differences between initial and final HPLC chromatogram area values for imprinted (Fig. S5A) and non-imprinted (Fig. S5B) composite cryogels were obtained from HPLC chromatograms versus different EV concentrations ($C = 1.0 \times 10^7$, 2.5×10^7 , 5.0×10^7 , 1.0×10^8 , 2.5×10^8 , 5.0×10^8 particles/mL). When these graphs were examined, it was observed that HPLC chromatogram area values increased with increasing EV concentration for the initial solutions. On the other hand, it was observed that for a difference between the initial and final EV solutions, HPLC chromatogram area values increased for a while with increasing EV concentration and then became saturated and stabilized. These graphs show that the imprinted composite cryogel exhibits increasing efficiency with increasing concentration. After a certain point, it reaches saturation and maintains a constant efficiency. Finally, when the area difference data for the imprinted and non-imprinted composite cryogels compared it can be deduced that efficient imprinting process recorded.

3.5. Benchmarking of EV isolation and purity validation

To benchmark the performance of the MIP-GO composite cryogel system against conventional ultrafiltration (UF) and ultracentrifugation (UC), both NTA and Western blot assays were conducted. NTA measurements distinctly demonstrated the disparities in particle dispersion and selectivity among the approaches. The stock sample had an exceedingly high particle concentration (2.7×10^{11} particles/mL) and a broad, multimodal distribution, signifying a complex mixture of EV and other nanoparticles (Fig. 6A). In the UF fraction (3.2×10^8 particles/mL), two distinct peaks were detected (Fig. 6B). The UC fraction (2.5×10^8 particles/mL) exhibited a predominant EV-sized peak alongside minor subpopulations, aligning with the established variability of centrifugation-derived isolates that may encompass microscopic microvesicles or protein aggregates (Fig. 6C). In the MIP-GO composite

cryogel, the initial fraction (3.6×10^8 particles/mL) maintained a profile similar to the stock (Fig. 6D), whereas the final fraction (2.4×10^8 particles/mL) displayed a pronounced unimodal peak within the EV size range (~ 130 nm), thereby confirming the effective elimination of larger and smaller impurities and signifying the selective retention of EVs within the cryogel (Fig. 6E). The desorbed fraction (4.7×10^7 particles/mL) exhibited a decreased concentration yet an EV-consistent, wider peak, indicating the regulated release of specifically trapped vesicles from the imprinted cavities rather than non-selective elution (Fig. 6F). The particle concentration in the MIP-GO desorbed fraction (4.7×10^7 particles/mL) corresponds to a controlled recovery of selectively captured EVs, representing the intentional elution of bound vesicles rather than non-specific release. This demonstrates the system's ability to recover target EVs with minimal loss or damage. Clear, unimodal distribution in the MIP-GO final fraction contrasts with the broad, multimodal UF profile and the slightly heterogeneous UC fraction. This indicates superior separation efficiency, as the cryogel selectively retains EVs within its imprinted cavities and eliminates impurities through controlled flow conditions.

In line with the 2018 MISEV (Minimal Information for Studies of Extracellular Vesicles) guidelines [45], the isolated EVs were thoroughly characterized using three complementary techniques: Western blotting to confirm protein markers, NTA to determine size distribution, and electron microscopy to examine their morphology [6,46,47]. Briefly, Western blot analysis corroborated the NTA results. CD63, a standard extracellular vesicle marker, was identified in all purified fractions (UF, UC, MIP-GO), while Calnexin, a negative control for cellular contamination, was present alone in the stock sample. The lack of Calnexin upon enrichment verifies elevated EV purity and negligible cellular debris. These results collectively indicate that MIP-GO composite cryogels attain purity and size specificity comparable to UC, while offering a label-free, reusable, and gentle separation method. Compared to UF and UC, the MIP-GO composite cryogels demonstrates enhanced selectivity and regulated capture–release capabilities, highlighting its promise as a reliable platform for EV separation and characterization. Collectively, these results demonstrate that the MIP-GO composite cryogel platform achieves UC-comparable purity, effective and selective recovery, and superior separation efficiency compared to UF and UC, providing a reproducible, label-free, and gentle alternative for EV isolation.

4. Conclusion

EVs serve as crucial intercellular communication mediators in cancer, facilitating the exchange of biomolecular information. However, the absence of reliable, reproducible, and user-friendly platforms for their efficient isolation and detection remains a significant challenge. This

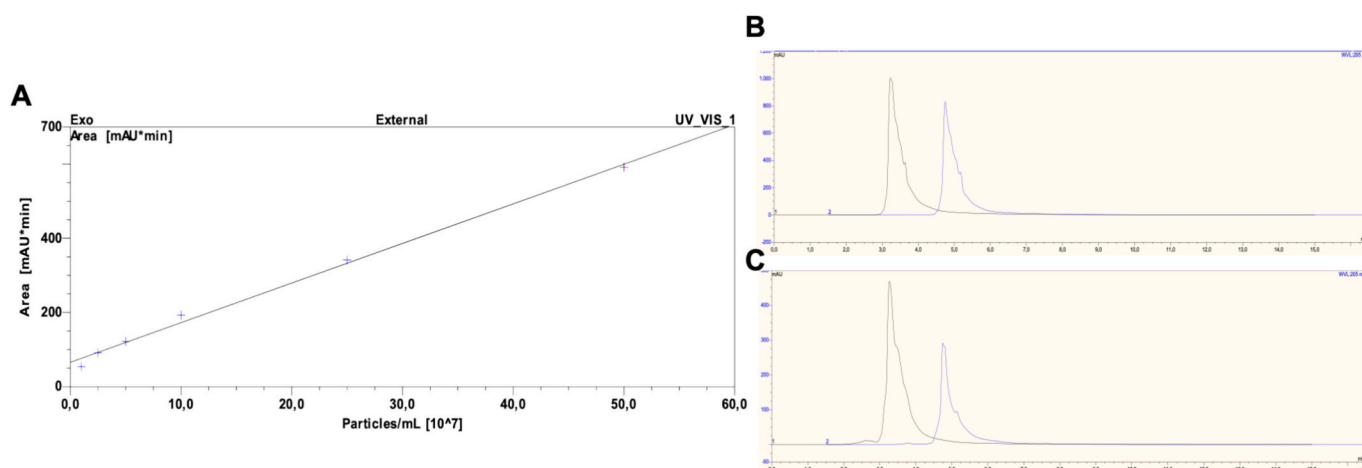


Fig. 5. Calibration curve (A), HPLC chromatograms for imprinted (B) and non-imprinted (C) composite cryogels.

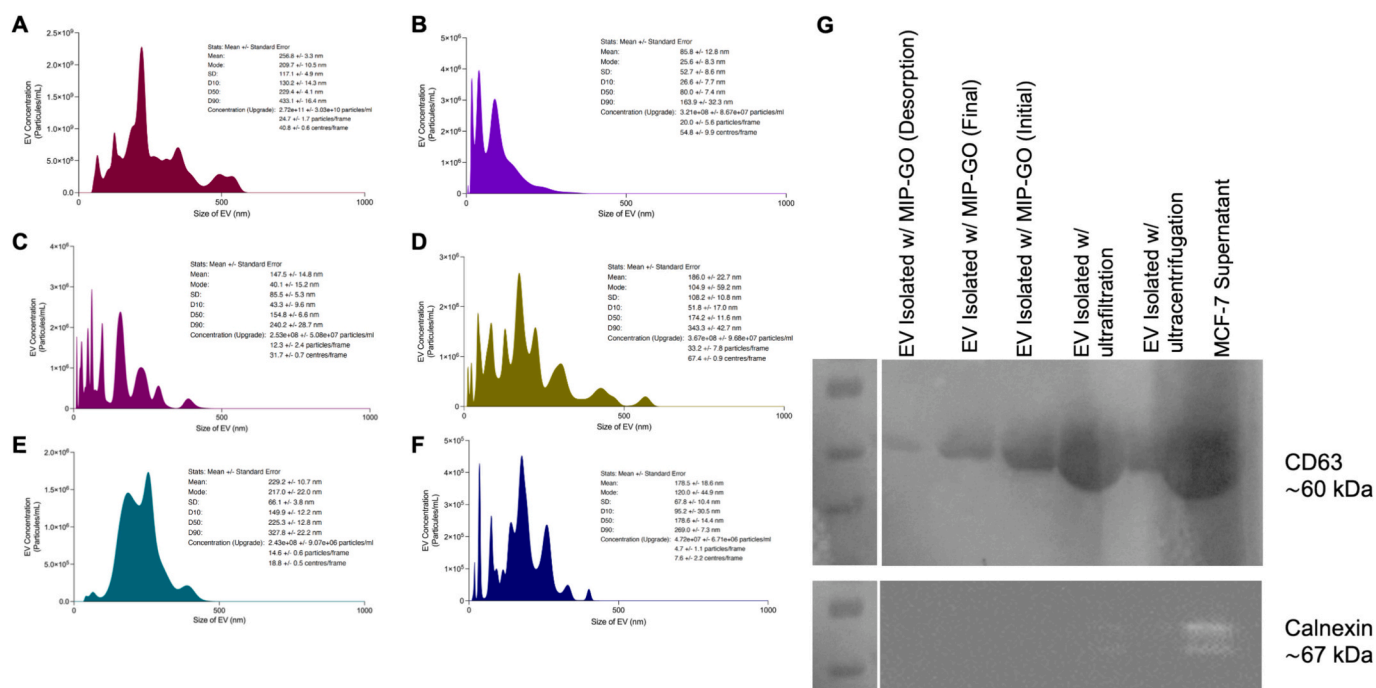


Fig. 6. NTA size-distribution profiles of EVs obtained from MCF-7 stock supernatant (A), ultrafiltration (B), ultracentrifugation (C), MIP-GO initial fraction (D), MIP-GO final fraction (E), and MIP-GO desorbed fraction (F). Western blot analysis of the EV marker CD63 and negative control Calnexin (G).

study introduces the preparation and comprehensive characterization of GO-cellulose composite cryogels specifically engineered for the selective and efficient isolation of EVs. The cryogels were fabricated using molecular imprinting technology, enabling the creation of precise binding sites for EVs. These materials exhibit remarkable adsorption capacity, exceptional selectivity, and robust stability. The synthesis process is efficient, reliable, and cost-effective, rendering it highly suitable for incorporation into polymer-dependent platforms for isolation, adsorption, and separation applications. Notably, this study represents the first reported integration of molecular imprinting with graphene oxide-cellulose for EV isolation, achieving a higher EV adsorption capacity of 231 particles/g for the composite cryogel. This enhancement in performance and selectivity is compared to conventional polymer-based systems. Additionally, the selective EV adsorption study was validated through HPLC studies, confirming that the imprinting process was performed successfully. Moreover, this innovative approach demonstrates adaptability for the adsorption of other biomolecules, offering a promising framework for the development of next-generation biomaterials characterized by high specificity, superior selectivity, and excellent reusability in diverse adsorption systems.

Compared to conventional EV isolation methods, our approach demonstrates distinct advantages in selectivity, efficiency, and physiological relevance. In brief, traditional UC and size-exclusion chromatography, while widely utilized as standardized methods, are often limited by low throughput, lengthy processing times, and potential co-isolation of contaminants, and structural damages on EVs. Polymer-based precipitation methods, on the other hand, suffer from non-specificity and may potentially contaminate EV membrane and proteins, and immunoaffinity-based approaches, although highly selective, require prior knowledge of EV surface markers and are constrained by capacity (low-throughput) and cost. In contrast, this study demonstrates a label-free, highly selective capture of EVs, leveraging molecular imprinting to create target-specific recognition sites. Integration with microfluidic tumor-mimicking cultures further ensures that the isolated EVs reflect physiologically relevant characteristics. Comprehensive characterization and kinetic studies under varying environmental parameters confirm the robustness and efficiency of this study.

Collectively, these features position our methodology as a versatile and powerful tool for EV isolation, offering both practical scalability and high fidelity in capturing biologically relevant vesicles, surpassing the limitations of existing approaches. In summary, our methodology presents several key advancements: (i) integration of microfluidics, allowing EVs to be derived from a dynamic, tumor-mimicking environment that more closely resembles *in vivo* conditions than standard static cultures; (ii) the use of GO-cellulose cryogels, which provide high surface area, mechanical stability, and enhanced adsorption properties; (iii) application of molecular imprinting to create EV-specific cavities, improving selectivity beyond traditional antibody-based approaches without requiring specific markers; (iv) comprehensive kinetic optimization across pH, temperature, ionic strength, and flow rate, ensuring robust performance under diverse conditions; and (v) validation via HPLC, providing quantitative confirmation of EV capture and release efficiency, a feature often absent in conventional methods.

CRedit authorship contribution statement

Yeşeren Saylan: Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Özge Altıntaş:** Writing – original draft, Validation, Methodology, Data curation. **Eylül Gulsen Yilmaz:** Writing – original draft, Methodology, Data curation. **Garbis Atam Akceoglu:** Writing – review & editing, Methodology, Data curation. **Fatih İnci:** Writing – review & editing, Supervision, Resources, Conceptualization. **Fatma Yılmaz:** Writing – review & editing, Validation, Data curation. **Adil Denizli:** Writing – review & editing, Validation, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2025.116029>.

Data availability

Data will be made available on request.

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