(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 April 2020 (30.04.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/086471 A1

(51) International Patent Classification:
B03C 1/30 (2006.01) *G01N 33/48* (2006.01)
B03C 1/02 (2006.01) *G01N 33/00* (2006.01)

(21) International Application Number:
PCT/US2019/057237

(22) International Filing Date:
21 October 2019 (21.10.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/748,470 21 October 2018 (21.10.2018) US

(71) Applicants: **THE UNIVERSITY OF KANSAS** [US/US]; 245 Strong Hall, 1450 Jayhawk Boulevard, Lawrence, KS 66045 (US). **KANSAS STATE UNIVERSITY RESEARCH FOUNDATION** [US/US]; 2005 Research Park Circle, Manhattan, KS 66502 (US).

(72) Inventor: **HE, Mei**; 11532 Windsor Street, Overland Park, KS 66224 (US).

(74) Agent: **COOK, Crissa, A.**; Hovey Williams LLP, 10801 Mastin Blvd, Suite 1000, 84 Corporate Woods, Overland Park, KS 66210 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH,

WO 2020/086471 A1

(54) Title: METHODS FOR GENERATING THERAPEUTIC DELIVERY PLATFORMS

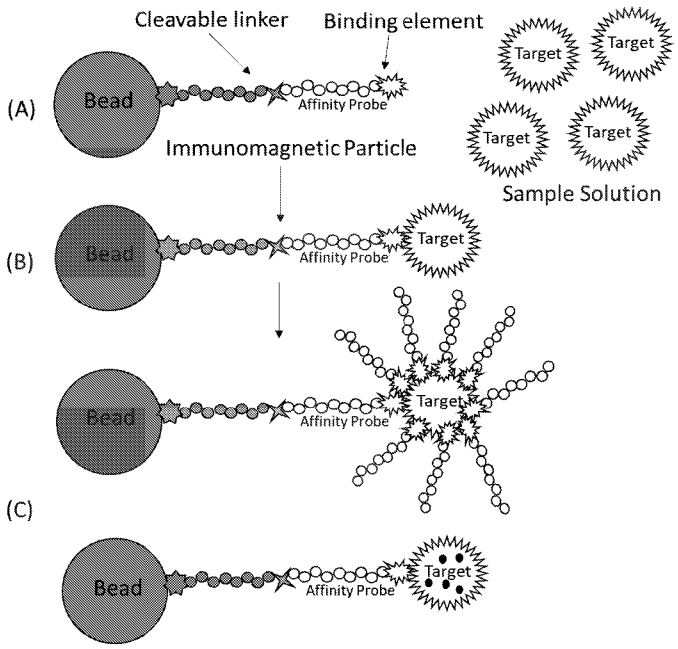


FIG. 1

(57) Abstract: Methods for producing engineered exosomes and other vesicle-like biological targets in a microfluidic device, including allowing a target vesicle-like structure to react and bind with immunomagnetic particles; capturing the immunomagnetic particle/vesicle complex by applying a magnetic field; further engineering the captured vesicles by surface modifying with additional active moieties or internally loading with active agents; and releasing the engineered vesicle-like structures, such as by photolytically cleaving a linkage between the particle and engineered vesicle-like structures, thereby releasing intact vesicle-like structures which can act as delivery vehicles for therapeutic treatments.

[Continued on next page]

GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

METHODS FOR GENERATING THERAPEUTIC DELIVERY PLATFORMS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the priority benefit of U.S. Provisional Patent Application Serial No. 62/748,470, filed October 21, 2018, entitled MICROFLUIDIC ON-DEMAND CAPTURE, LOADING, AND PHOTO-RELEASE OF EXTRACELLULAR VESICLES AND EXOSOMES AS VACCINE DELIVERY PLATFORM, incorporated by reference in its entirety herein.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under 2017-67021-26600 awarded by the USDA National Institute of Food and Agriculture and GM103638 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

The following application contains a sequence listing in computer readable format (CRF), submitted as a text file in ASCII format entitled "Sequence_Listing," created on October 21, 2019, as 12 KB. The content of the CRF is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The disclosure generally relates to microfluidic devices and methods for harvesting intact carriers or delivery vehicles for delivery of bioactive therapeutics, such as peptides or proteins, nucleotides, and other active agents (e.g., chemicals and drugs).

Description of Related Art

Among all deliverable cells and nanoparticles, live-cell derived extracellular vesicles, especially exosomes in the nano-size range of 30~150 nm, have shown important roles in intercellular communications in recent decades. The immune cell-derived exosomes have been well documented in the regulation of immune stimulation or suppression, driving inflammatory, autoimmune and infectious disease pathology. The formation of exosomes begins with the creation of endosomes as the intracellular vesicles. Exosomes are differing from other membrane-derived microvesicles by originating from multivesicular bodies (MVBs) for cellular secretion. Therefore,

exosomes contain specific proteins and nucleic acids and represent their parent cell status and functions at the time of formation in parent cells. Among many subtypes of exosomes, the immunogenic exosomes with an intrinsic payload of MHC class I and II molecules and other co-stimulatory molecules are able to mediate immune responses, which opens up opportunities for the development of novel delivery platforms which can be used for cancer vaccines, immunotherapy delivery, and other delivery associated with *in vivo* transportation.

Compared to other nano-sized delivery systems, such as lipid, polymers, gold and silica material, exosomes are living-cell derived, highly biocompatible nano-carriers with intrinsic payload, and exhibit much stronger flexibility in loading desired antigens for effective delivery. Exosomes also eliminate allergenic responses without concerns of carrying virulent factors and avoid degradation or loss during delivery. However, the development of exosome-based vaccines is hindered by substantial technical difficulties in obtaining pure immunogenic exosomes. The diverse subtypes of exosomes could confound the investigation on differentiating different cellular messages. On the other hand, molecular engineering of exosomes through either membrane surface or internal loading could provide an untapped source for developing novel antigenic exosomes.

Bioengineered exosomes as emerging delivery vehicles have gained substantial attention in developing a new generation of cancer vaccines, including recent phase-II trial using IFN-DC-derived exosomes loaded with MHC I/II restricted cancer antigens to promote T cell and natural killer (NK) cell-based immune responses in non-small cell lung cancer patients. Unfortunately, current exosome engineering approaches, such as the transfection or extrusion of parent cells, and membrane permeabilization of secreted exosomes, suffer from poor yield, low purity, and time-consuming operations. There is a need for methods of producing exosomes to solve this bottleneck problem. Due to the intrinsic features in automation and high-efficient mass transport, microfluidic systems overcome many drawbacks of benchtop systems and show superior performance in isolating, detecting and molecular profiling of exosomes. However, molecular engineering of exosomes using microfluidic platform has not been explored. Presently, the most reported work on processing exosomes is either in small quality or bound to solid surface/particles, and they are unable to stay intact for downstream therapeutic preparations.

SUMMARY

Methods and microfluidic devices are described herein to engineer a variety of biologic carriers or delivery vehicles, such as cells, extracellular vesicles and exosomes, and membrane or lipid particles, and polymer particles. These carriers can be captured using the inventive methods and devices, loaded with active agents (either surface modified or encapsulated), and then released as intact, engineered carriers for delivering a variety of therapeutic compounds and bioactive agents. The engineered carriers can be used for diagnostics, prognostics, companion assays, pharmaceutics and therapeutics, immunotherapy and vaccine delivery, and tissue delivery, and other usage associated with *in vivo* transportation of active agents. Embodiments described herein are exemplified with respect to exosomes. However, it will be appreciated that exosomes represent a particularly challenging biological target, such that it is envisioned that the platform can be applied to other similar structures—vesicular or vesicle-like structures characterized by a liquid core and membrane or bilayer—including cells (including T-cells), microsomes, and the like. These biological targets, which are then engineered into carriers or delivery vehicles can also be characterized as nanocarriers.

Provided herein are microfluidic analytical devices and method of on-demand capture, loading, and photo-release of intact engineered nanocarriers. The microfluidic devices can enable real-time harvesting and antigenic modification of extracellular vesicles, particularly exosomes, with subsequent release of intact exosomes downstream on-demand. Also disclosed are magnetic-nanoparticles functionalized with photo-cleavable, affinity probes (active moieties) for capturing and on-demand releasing MHC-I positive exosomes via a light trigger. The affinity probe can include an antigenic peptide, antibody, aptamer, nanobody and other affinity-based probes. The photo-release of the modified/loaded exosomes in the microfluidic devices can be well controlled spatially and temporally with 95% or greater efficiency. Such a functional streamlined microfluidic cell culture system allows antigenic engineering of exosomes either through mediating their parent cell growth using stimulations, or direct molecular engineering on the surface of produced exosomes. Heterogeneity of exosome subtypes has been found from the same population of parent cells. The released subtypes of exosomes contain distinct molecular and biological properties for different cellular regulation. The disclosed methods can capture, load, and release specific subtype of exosomes with more targeted therapeutic functions. The carriers that can be used in this disclosed method for capture, loading and release include cells, extracellular vesicles and exosomes, and membrane or lipid particles, and polymer particles, which can encapsulate drugs (small molecule compounds), genes and bioactive therapeutics. Proof of concept with several

tumor antigenic peptides (e.g., gp-100, MAGE-A3, and MART-1) which are commonly used in developing cancer vaccines but difficult in delivery due to the degradation have been demonstrated herein. The microfluidic devices show high-efficiency in engineering immunogenic exosomes (MHC I+), meanwhile, photo-releasing the intact functional exosomes downstream. Cellular uptake of engineered exosomes by antigen presentation cells has also been demonstrated, which displayed much-improved internalization ability compared to non-engineered exosomes. In particular, the engineered exosomes show significantly higher activation rate (at least 30%) for activating T cells by challenging CD8 T cells purified from the spleen of 2 Pmel1 transgenic mice, than non-engineered exosomes. We also assessed the degree of potency of antimicrobial peptide-engineered immunogenic exosomes for stimulating T cells *ex vivo* using transgenic mice for treating bovine respiratory syncytial virus (BRSV) infections. Exosomes engineered with BRSV targeting peptide (Peptide 4: M187-195 peptide NAITNAKII, SEQ ID NO:4) have the capacity to activate BRSV M-specific T cells in the presence of activated dendritic cells.

Accordingly, the engineered exosomes are viable and functional for application in cancer immunotherapy and vaccination for infectious disease. The facile and low-cost microfluidic platform for producing engineered vesicles not only provides an enabling strategy for high-efficiency production of purified, enriched therapeutic vesicles but also serve as an investigation tool for understanding roles of variable peptide-engineered exosomes in antitumor immune responses, cancer immunotherapy, and vaccination for treating infections.

In specific aspects, the microfluidic devices disclosed herein can comprise a cell culture chamber dimensioned to maintain biological material in a three-dimensional configuration; a mixing channel fluidly connected to the cell culture chamber and comprising a plurality of sample inlet channels disposed along the mixing channel, wherein the ratio of a width of the cell culture chamber to the largest cross-sectional dimension of the mixing channel is at least 5:1; an isolation channel defining a path for fluid flow from the mixing channel to an isolation outlet; and a collection chamber fluidly connected to the isolation outlet and comprises a magnet operatively coupled to the collection chamber to produce a magnetic field within the collection chamber.

In other aspects, the microfluidic devices can comprise a cell culture chamber comprising a cell culture inlet and a cell culture outlet; a fluid inlet channel and a particle inlet channel, wherein the cell culture outlet, the fluid inlet channel, and the particle inlet channel fluidly converge at a mixing intersection; a mixing channel fluidly connected to the mixing intersection and defining a path for fluid flow from the mixing intersection to a mixing outlet, wherein the ratio of a width of the cell culture chamber to the largest cross-sectional dimension of the mixing channel is at least

5:1; and a collection chamber fluidly connected to the mixing outlet and comprises a magnet operatively coupled to the collection chamber to produce a magnetic field within the collection chamber. In these aspects, the mixing channel can comprise an isolation channel disposed between the mixing intersection and the mixing outlet.

The isolation channel in the microfluidic devices can have a geometry to induces turbulent flow so as to mix flowing fluids in the device. For example, the isolation channel in the microfluidic devices can have a serpentine geometry. The isolation channel can further include one or more channel constriction domain that decreases in width for producing a local vortex flow profile. In certain embodiments, the isolation channel can comprise a plurality of channel constriction domains, preferably at least 5 channel constriction domains.

As described herein, the microfluidic devices comprise a cell chamber and a mixing channel. The cell chamber and the mixing channel can have a height and a width. The height and the width of the mixing channel can each be at least 50 microns, preferably between 50 and 500 microns. The ratio of the cell culture chamber width to the largest cross-sectional dimension of the mixing channel can be from 5:1 to 500:1, from 5:1 to 200:1, from 5:1 to 100:1, from 5:1 to 20:1, preferably from 6:1 to 12:1. The cell culture chamber can have a volume of about 200 microliters or greater, preferably from about 200 microliters to about 1 milliliter.

The microfluidic devices disclosed herein can further comprise a pump operably coupled to the device.

It also disclosed the method of using the device for capture of a target in a sample solution, loading, and on-demand photo release can be used for harvesting intact delivery carriers. Such carriers can be cells, extracellular vesicles and exosomes, and membrane or lipid particles, and polymer particles, which can encapsulate drugs, genes and bioactive therapeutics. Specifically, this integrated and continuous method of capture, loading and photo-release can produce the engineered exosomes in a microfluidic device. The methods comprise introducing a biological sample containing exosomes (or another target) into a mixing channel and mixing the exosomes with immunomagnetic particles and a wash buffer to form a mixture; allowing the exosomes to react and affinity bind with the immunomagnetic particles; and collecting the exosomes bound to the immunomagnetic particles by applying a magnetic field within a collection chamber. The method for producing the engineered exosomes can include introducing cells into a cell culture chamber of the microfluidic device and first culturing the cells under conditions allowing the release of exosomes. This can be carried out in-line, in the same microfluidic device used for capture and loading.

The cells from which the exosomes are released can be selected from dendritic cells, stem cells, immune cells, megakaryocyte progenitor cells, macrophages, or other live cells.

The immunomagnetic particles bound to the exosomes can comprise a magnetic particle-bound to an affinity probe for capturing exosomes via a moiety comprising a photocleavable linker. As described herein, the affinity probe for capturing exosomes can include an antigen peptide, antibody, aptamer, or antigenic epitope thereof for capturing the exosomes. Suitable examples of antigen peptides include MAGE-A3, gp-100, HER-2, p53, PSA-1, or MART-1. The moiety comprising the photocleavable linker can include biotin bound to immunomagnetic particle and attached at the other end via a photocleavable linker to the affinity probe. The affinity probe targets surface proteins on the target (e.g., immunostimulatory molecules or markers). Suitable immunostimulatory molecules include an MHC class I molecule, an MHC class II molecule, an interleukin, TNFα, IFNγ, RANTES, G-CSF, M-CSF, IFNα, CTAPIII, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1β and combinations thereof. Preferably, the affinity probe is itself an antigenic moiety (peptide) which preferentially binds to a surface protein on the target.

After mixing, the immunomagnetic particles capture/bind exosomes (or other target) in the sample. The immunomagnetic particles are immobilized in the device, e.g., using a magnet positioned adjacent to a collection chamber. The immobilized bead/exosome complexes are then washed and incubated with buffer solution containing active moieties for either surface loading onto the captured exosomes or internal encapsulation, as described in more detail below. The methods can further comprise photolytically cleaving the captured, modified exosomes from the immunomagnetic particles, releasing intact, engineered exosomes comprising the active agent (antigen peptide or antigenic epitope thereof).

The methods for producing the engineered biological targets, such as exosomes, can be performed using the microfluidic devices disclosed herein. In some embodiments, the methods are carried out in real-time. In one or more embodiments, the methods are streamlined (aka "continuous"), such that the capture, loading, and release of the biological target occurs in the same device/container (i.e., without having to transfer or move between containers or reaction tubes, but rather in-line along a microfluidic channel and in-line capture/engineering chamber). Thus, each of the steps can be carried out consecutively, one after the other using the various inlets which converge at a single microfluidic channel, and preferably substantially immediately one after the other. In other words, the method involves immunomagnetic bead loading, followed immediately or nearly simultaneously with sample loading, followed by loading of the active agents to be attached to or loaded into the captured target. Each component loaded into the

microfluidic device converges from respective inlets into a single microfluidic channel, followed by "automatic" retention in the capture/engineering chamber downstream from the inlet by the magnet positioned adjacent to the chamber. As the fluid mixture flows through the channel and then the chamber, the respective reactions are occurring in real-time (i.e., capture and loading). Application of light to the chamber can then release the engineered target. It will be appreciated that the streamlined process is much faster than traditional benchtop methods. Preferably, the process from sample/bead loading at respective inlets to collection of the released at the outlet, engineered target can be completed within approximately 2 hours total, more preferably within approximately 90 minutes, and more preferably within approximately 1 hour.

Compositions comprising engineered exosomes, particularly immunogenic exosome complexes are also disclosed. The immunogenic exosome complex can comprise an antigenic peptide or antigenic epitope thereof conjugated to a surface of an exosome, wherein the immunogenic exosome complex activates T-cell by at least 30% compared to a native exosome. The antigenic loading of such antigenic peptide or antigenic epitopes can be performed after mixing and capture exosomes for completely coating exosome surface with antigenic peptides. The compositions can be prepared using the methods disclosed herein. Accordingly, immunogenic exosome complex prepared by a process comprising introducing cells into a cell culture chamber of a microfluidic device; culturing the cells under conditions allowing release of engineered exosomes; introducing the engineered exosomes into a mixing channel and mixing the engineered exosomes with immunomagnetic particles and a wash buffer to form a mixture for exosome capture/binding; then apply a magnetic field within a collection chamber to collect the isolated exosomes, allowing to react with the loading buffer containing antigenic peptides to form an immunogenic exosome complex; and apply UV light to break the photo-cleavable linker for collecting the immunogenic exosome complex at the outlet of microfluidic device.

Pharmaceutical compositions comprising the immunogenic exosome complex are also disclosed. The loading targets can be drugs, genes and bioactive therapeutics. Methods of treating disease in a subject comprising administering to the subject a pharmaceutical composition comprising an immunogenic exosome complex are disclosed. In certain embodiments, the disease can be an infection. In some examples, the disease can be cancer. When the disease to be treated is cancer, the methods can further include administering a chemotherapeutic agent.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Fig. 1 illustrates (A) immunomagnetic beads mixed with a sample solution, (B) capture of targets in a sample solution using immunomagnetic beads; and (C) loading of antigenic or active agents onto or inside of the captured target.

Fig. 2A illustrates (A) application of light or activating radiation to the bead/target complex, and (B) photorelease of an engineered target (carried) surface modified with active agents or antigenic moieties.

Fig. 2B illustrates (A) application of light or activating radiation to the bead/target complex, and (B) photorelease of an engineered target (carrier) loaded with active agents inside.

Fig. 3 is an illustration of a process overview for a 3D-printed molded PDMS microfluidic culture chip for streamlined engineering of antigenic exosomes employed in activating anti-tumor responses.

Fig. 4 illustrates an embodiment of a microfluidic device.

Fig. 5 shows images of (a) a microfluidic channel and flow; (b) mixing with microbeads; (d) morphology of cells; and (e) SEM image of released exosomes.

Fig. 6 illustrates an embodiment of a microfluidic device.

Fig. 7A shows an illustration of immunomagnetic capture and on-demand photo-release of MHC-I positive, immunogenic exosomes.

Fig. 7B shows characterization of three tumor-targeting peptide antigens conjugated with photo-cleavable immunomagnetic beads for binding and photo-release of fluorescence-labeled immunogenic exosomes. The MHC-I antibody is used as the positive control to compare the binding strength between MHC-I positive exosomes and tumor targeting peptides.

Fig. 8A show characterization of the performance of on-demand photo-release of captured exosomes from immunomagnetic capture beads. The positive control is a fluorescence-labeled antibody captured by photo-release immunomagnetic beads. The negative control is the immunomagnetic beads without a photo-cleavable linker.

Fig. 8B shows the SEM image of a surface of photo-release immunomagnetic beads captured with exosomes. Exosome particles were seen as the cup shape due to the vacuum sample preparation.

Fig. 8C shows the SEM image of the surface of photo-release immunomagnetic beads after photocleavage.

Fig. 8D shows characterization of UV exposure time influence on the photo-cleavage efficiency.

Fig. 8E shows nanoparticle tracking analysis of exosome size distribution between engineered exosomes and non-engineered exosomes.

Fig. 9A shows the confocal microscope images of DC uptake of tumor targeting antigenic (TTA) peptide, gp-100 surface engineered exosomes, compared with non-engineered exosomes. The image was taken every hour for tracking the green fluorescence labeled exosomes uptake by DCs (cell nuclei were stained with DAPI).

Fig. 9B shows the release of cytokine IFN-γ from DCs culture measured by ELISA for monitoring 48 hours, compared between non-engineered exosomes and gp-100 engineered exosomes.

Fig. 10A depicts representative flow plots from wells containing T cells + activated JAWS cells with increasing concentrations of the gp100-engineered exosomes.

Figure 10B depicts the cumulative data from all three culture conditions showing the CD8+ T cell dividing rate under stimulation. The results are representative of 2 independent experiments with duplicate wells for each culture condition.

Fig. 11 depicts flow cytometry plots from wells containing T cells and activated JAWS cells with increasing concentrations of the bovine respiratory syncytial virus (BRSV) antimicrobial peptide-engineered exosomes for depicting the immunogenic potency.

Fig. 12A illustrates a 3D printing approach for producing 3D mold integrated with cell culture and downstream exosome isolation, surface engineering, and on-demand photo release.

Fig. 12B shows the results from replicating PDMS microfluidic device.

Fig. 13 shows results from investigation of the side-effect of UV exposure on exosome molecular contents in terms of proteins, DNAs and RNAs.

Fig. 14 shows dendritic monocytes culture under different stimulation conditions: dendritic monocytes without any stimulation (negative control; first image); PWM protein stimulation (positive control; second image); and gp-100 engineered exosome stimulation (last image).

DETAILED DESCRIPTION

The following description of the disclosure is provided as an enabling teaching of the disclosure in its best, currently known embodiment(s). To this end, those skilled in the relevant art

will recognize and appreciate that many changes can be made to the various embodiments of the invention described herein, while still obtaining the beneficial results of the present disclosure. It will also be apparent that some of the desired benefits of the present disclosure can be obtained by selecting some of the features of the present disclosure without utilizing other features. Accordingly, those who work in the art will recognize that many modifications and adaptations to the present disclosure are possible and can even be desirable in certain circumstances and are a part of the present disclosure. Thus, the following description is provided as illustrative of the principles of the present disclosure and not in limitation thereof.

Terminology

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. The following definitions are provided for the full understanding of terms used in this specification.

Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular fluidic channel is disclosed and discussed and a number of modifications that can be made to the fluidic channels are discussed, specifically contemplated is each and every combination and permutation of the fluidic channels and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of fluidic channels A, B, and C are disclosed as well as a class of fluidic channels D, E, and F and an example of a combination fluidic channels, or, for example, a combination fluidic channels comprising A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

It is understood that the devices disclosed herein have certain functions. Disclosed are certain structural requirements for performing the disclosed functions, and it is understood that there are a variety of structures which can perform the same function which are related to the disclosed structures, and that these structures will ultimately achieve the same result.

Unless otherwise expressly stated, it is in no way intended that any method set forth herein be construed as requiring that its steps be performed in a specific order. Thus, where a method claim does not expressly recite an order of steps to be followed or it is not otherwise specifically stated in the claims or description that the steps are to be limited to a specific order, it is no way intended that an order be inferred, in any respect. This holds for any possible non-express basis for interpretation, including: matters of logic with respect to arrangement of steps or operational flow; plain meaning derived from grammatical organization or punctuation; and the number or type of embodiments described in the specification.

As used in the specification and claims, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "an agent" includes a plurality of agents, including mixtures thereof.

As used herein, the terms "can," "may," "optionally," "can optionally," and "may optionally" are used interchangeably and are meant to include cases in which the condition occurs as well as cases in which the condition does not occur. Thus, for example, the statement that a formulation "may include an excipient" is meant to include cases in which the formulation includes an excipient as well as cases in which the formulation does not include an excipient.

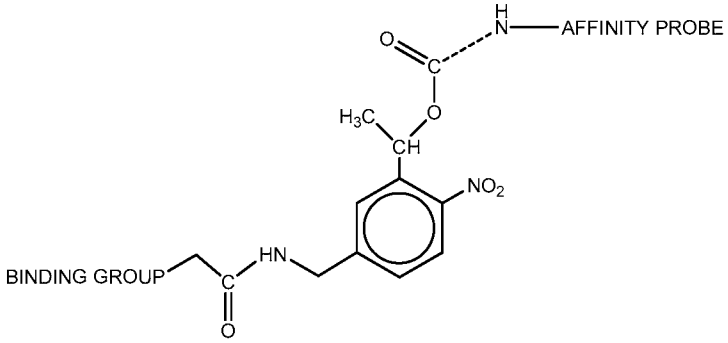
Ranges can be expressed as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It is also understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed.

The terms "upstream" and "downstream" refer to positions within a device which are relative another position and a direction of fluid flow. As used herein, the term "upstream" refers to a first position that is located in a direction opposite the direction of fluid flow relative to a second position. Conversely, as used herein, the term "downstream" refers to a second position

that is located in a direction along the direction of fluid flow relative to a first position.

Methods

With reference to Fig. 1, the general methods described herein involve a plurality of immunomagnetic particles (beads), which are mixed with a sample solution suspected of containing the target. In some embodiments, a biological sample is collected from a subject and prepared for the method, e.g., by diluting with buffer, concentrating, etc. In some embodiments, cells are collected and expanded in culture. In some embodiments, cells are collected and cultured under conditions to release exosomes or other extracellular vesicles or vesicle-like structures into culture. In any event, the immunomagnetic particles contain a photocleavable linker and affinity probe (and preferably a plurality of photocleavable linkers, each with a respective affinity probe) extending from the particle surface for capturing the target. The immunomagnetic particles are contacted with a sample solution for a period of time sufficient for the target (if present in the sample) to interact with the affinity probes extending from the immunomagnetic particles. In Fig. 1, a single bead is depicted with a single linker for ease of illustration; however, in practice, each immunomagnetic particle will be coated with a plurality of linkers (preferably substantially the entire surface area of the particle/bead is coated with linkers). Moreover, the relative sizes in Fig. 1 are not to scale, but enlarged for illustration purposes. In practice, the bead/particle is preferably at least 5 times larger than the target (e.g., in the case of exosomes, which range 30-150 nm in diameter, the bead is preferably 500 nm or larger). In this way, a plurality of targets (e.g., exosomes) will be captured on the surface of a single bead/particle. Fig. 1 uses a single bead and target interaction for ease of reference. As shown in Fig. 1(A), the bead and sample solution are mixed for a sufficient period of time (e.g., in a mixing chamber or channel in the microfluidic device). If the target is present in the sample solution, it will be captured by the bead (and specifically by an affinity probe extending from the bead via its photocleavable linker), as illustrated in Fig. 1(B). Exemplary photocleavable linkers are described herein, and may include linear chains including biotin or similar moiety at one end for attachment to the particle and an amine moiety at the other end for attachment to the affinity probe. A preferred linker has the following structure, where the dashed line indicates the bond cleaved during photo exposure, and the "binding group" represents the moiety (e.g., biotin) used to attach the linker to the bead (directly or via a functionalized surface coating, e.g., avidin):



Although various embodiments are described herein, the affinity "probe" is typically an oligopeptide sequence that has specificity for and recognizes the target, such as a peptide that recognizes and acts as a receptor for a surface protein on the target. As used here, the phrase "specificity for" is intended to differentiate the affinity probe from non-specific binding or reactions between molecules, and means that the set of specific targets for which the affinity probe can interact is limited, and in some cases even exclusive, such that binding does not occurs at an appreciable rate with any other molecule except for the target (and specifically, its designated surface protein(s)). Short oligopeptide sequences are preferably used for the affinity probe including sequence segments with high specificity for the target. More preferably, upon binding, the affinity probe and surface protein create a complex that enhances the immunogenic potential of the target, as described in more detail herein and demonstrated in the working examples.

The bead with the captured target (e.g., exosome) is immobilized in the microfluidic device. The bead can be immobilized before or after capture of the target. As described elsewhere herein, this can be achieved by positioning a magnet adjacent a collection or engineering chamber in the microfluidic device. As the sample solution and bead solution flow through the microfluidic channel, the beads and target interact thereby capturing the target. The magnetic beads are immobilized in the collection or engineering chamber (thereby also immobilizing the captured target) as the solutions flow through. As illustrated in Fig. 1(C), the captured target is then engineered either by attaching a plurality of active agents (e.g., antigenic peptides) to the target surface or loading the target with drugs, chemicals, nucleotides, or other bioactive agents (e.g., CRISPR Cas9). For surface modification, the immobilized bead/target complex is washed and incubated in the microfluidic device with buffer solution containing a plurality of active agents having at least one moiety that has specificity for a surface protein presented on the surface of the target. Preferably, the active agents or moieties for surface loading are of the same "type" of compound (e.g., comprise the same oligopeptide) selected for the affinity probe used in the immunomagnetic particles. The immobilized bead/target complex is incubated with the active

agents for a period of time sufficient for the active agents to interact with the captured target. Preferably, the active agent loading into the microfluidic device is at a concentration such that substantially the entire surface of the target is coated with active agents (e.g., preferably, substantially all of the target surface protein is bound by active agent). Instead of surface modification, Fig. 1(C) also depicts an alternative where active agents can be loaded into the target. This can be carried out by washing and incubating the immobilized bead/target complex in the microfluidic device with buffer solution containing active agents to be loaded, along with detergents or chemical transfection reagents to induce pore formation in the target for active agent loading, followed by washing with buffer to remove the reagents and close the pores. In either embodiment, excess active agent is then washed away leaving engineered target immobilized with the immunomagnetic bead.

With reference to Fig. 2A, the engineered target can then be released by exposing the immobilized bead/target complex to activating radiation (e.g., light) of the appropriate wavelength to cleave the photocleavable linker. This process releases the engineered target along with the affinity probe which remains bound to the target, which now acts as an engineered carrier or delivery vehicle for the active agents decorated on the surface of the target. Likewise, in Fig. 2B, the same photorelease process can be used to release the targets internally loaded with active agents. A wash buffer can be introduced into the microfluidic device to transport the released targets downstream for collection at the outlet of the microfluidic device. Advantageously, the light release step in embodiments of the invention is carried out with exposure times of 15 minutes or less, preferably about 13 minutes or less, more preferably about 12 minutes or less. As demonstrated in the working examples, approximately 100% of the captured target is preferably released/cleaved within about 10 minutes of exposure time.

It will be appreciated that since the magnetic beads are already immobilized in the engineering chamber, a separate step is not required to separate the target from the magnetic beads in the solution. Rather, upon exposure, the linker between the captured target and the bead is cleaved, thereby releasing the engineered targets, which flow downstream away from the immobilized beads to the outlet of the microfluidic device. The released targets, which have been engineered with the active moieties (aka the “engineered carrier”), can then be collected for analysis and therapeutic use from the outlet of the microfluidic device or otherwise directly diverted to a further chamber or collection device. It will be appreciated that the immunomagnetic beads can then be subsequently collected for re-use by removing the magnetic field from the microfluidic device, such that the immunomagnetic beads are no longer magnetically immobilized.

The beads can be washed downstream and collected from the outlet.

As noted, this process can advantageously take place in a microfluidic device, and as a continuous, integrated, in-line approach for isolating, capturing, engineering, and releasing intact targets, such as exosomes from a sample, as therapeutic carriers or delivery vehicles.

Devices

Extracellular vesicles (≤1 μm), particularly exosomes (30-150 nm), are the emerging cargo for mediating cellular signal transductions. However, standard benchtop methods (e.g., ultracentrifugation and filtration) lack the ability to process immunogenic exosomes specifically among other microvesicle subtypes, due to time-consuming (>10 h) and extremely tedious isolation protocols. The present disclosure addresses needs in the art by providing devices that introduce a streamlined microfluidic platform for harvesting, antigenic modification and photo-release of immunogenic extracellular vesicles and exosomes directly from on-chip cultured cellular media. These devices provide automatic and rapid cell-culture production of antigenic exosomes that can be used in immunotherapy such as cancer immunotherapy. The devices disclosed herein enables real-time harvesting and antigenic modification of exosomes with subsequent photo-release downstream on-demand, as depicted in the overview in Fig. 3.

Turning now to Fig. 4, disclosed herein is a microfluidic device (200) comprising a cell culture chamber (210) dimensioned to maintain biological material in a three-dimensional configuration; a mixing channel (220) fluidly connected to the cell culture chamber and comprising a plurality of sample inlet channels (222, 224, 226) disposed along the mixing channel, wherein the ratio of a width of the cell culture chamber (210) to the largest cross-sectional dimension of the mixing channel (220) is at least 5:1; an isolation channel (230) defining a path for fluid flow from the mixing channel (220) to an isolation outlet (234); and a collection chamber (240) fluidly connected to the isolation outlet (234) and comprises a magnet operatively coupled to the collection chamber to produce a magnetic field within the collection chamber (240).

Devices of the present disclosure can be described by sizes and comparisons of sizes (e.g., ratios) of components within the device. In some embodiments, the cell culture chamber (210) has a volume of about 200 microliters or greater (for example, 200 microliters or greater, 250 microliters or greater, 300 microliters or greater, 350 microliters or greater, 400 microliters or greater, 450 microliters or greater, 500 microliters or greater, 550 microliters or greater, 600 microliters or greater, 650 microliters or greater, 700 microliters or greater, 750 microliters or greater, 800 microliters or greater, 850 microliters or greater, 900 microliters or greater, 950

microliters or greater, or 1 milliliter or greater). In some embodiments, the cell culture chamber (210) has a volume of about 1000 microliters or less (for example, 950 microliters or less, 900 microliters or less, 850 microliters or less, 800 microliters or less, 750 microliters or less, 700 microliters or less, 650 microliters or less, 600 microliters or less, 650 microliters or less, 550 microliters or less, 500 microliters or less, 450 microliters or less, 400 microliters or less, 350 microliters or less, 300 microliters or less, 250 microliters or less, or 200 microliters or less). In some embodiments, the cell culture chamber (210) has a volume of from about 200 microliters to about 1 milliliter (for example, from 200 microliters to 900 microliters, from 200 microliters to 750 microliters, from 200 microliters to 500 microliters, from 300 microliters to 750 microliters, or from 350 microliters to about 500 microliters). In some embodiments, the cell culture chamber has a sufficient volume such that the top can be left open for applying a plug (such as a PDMS-made, finger-push plug) for fluid exchange and pushing the fluid to downstream collection channels.

In some embodiments, the cell culture chamber has a height and a width. The cell culture chamber can have a height of at least 500 microns (for example, 500 microns or greater, 600 microns or greater, 650 microns or greater, 700 microns or greater, 750 microns or greater, 800 microns or greater, 850 microns or greater, 900 microns or greater, 950 microns or greater, or 1000 microns of greater). In some embodiments, the cell culture chamber has a height of 1000 microns or less (for example, 950 microns or less, 900 microns or less, 850 microns or less, 800 microns or less, 750 microns or less, 700 microns or less, 650 microns or less, 600 microns or less, or 500 microns or less). In some embodiments, the cell culture chamber has a height of from 500 microns to 1000 microns (for example, from 600 microns to 1000 microns, from 750 microns to 1000 microns, or from 800 microns to 1000 microns).

The cell culture chamber can have a width of at least 200 microns (for example, 250 microns or greater, 275 microns or greater, 300 microns or greater, 350 microns or greater, 400 microns or greater, 450 microns or greater, 500 microns or greater, 550 microns or greater, or 600 microns or greater). In some embodiments, the cell culture chamber has a width of 1000 microns or less (for example, less than 1000 microns, 750 microns or less, less than 750 microns, 600 microns or less, 550 microns or less, or 500 microns or less). In some embodiments, the cell culture chamber has a width of from 250 microns to 1000 microns (for example, from 250 microns to 750 microns, from 250 microns to 500 microns, from 300 microns to 750 microns, or from 300 microns to 500 microns).

As described herein, the mixing channel (220) fluidly connects to the cell culture chamber

and comprises a plurality of sample inlet channels (222, 224, 226) disposed along the mixing channel. The plurality of sample inlet channels can include a cell culture inlet channel (also referred to herein as B-inlet, 222) that fluidly connects to the cell culture chamber and defines a path for introducing fluid from the cell culture chamber into the mixing channel. The plurality of sample inlet channels can further include a particle inlet channel (also referred to herein as A-inlet, 224) that defines a path for introducing particles into the mixing channel. The plurality of sample inlet channels can further include a fluid inlet channel (also referred to herein as C-inlet, 226) that defines a path for introducing fluid (such as a wash buffer) into the mixing channel. The plurality of sample inlet channels can be in any arrangement. For example, the cell culture inlet channel can be upstream of the particle inlet channel which is upstream of the fluid inlet channel. In other examples, the particle inlet channel can be upstream of the cell culture inlet channel which is upstream of the fluid inlet channel.

The cell culture inlet channel, the particle inlet channel, and the fluid inlet channel can fluidly converge at a mixing intersection. The cell culture inlet channel forms a path for fluid flow from the cell culture chamber to the mixing intersection. The particle inlet channel forms a path for fluid flow from a particle inlet to the mixing intersection. The fluid inlet channel forms a path for fluid flow from a fluid inlet to the mixing intersection. As used herein, a path of fluid flow can be represented pictorially in the figures by an arrow to indicate the direction of fluid flow through the path of fluid flow.

The mixing channel comprising the cell culture inlet channel, the particle inlet channel, and the fluid inlet channel has a height and a width. In some embodiments, the mixing channel has a height of at least 50 microns (for example, 75 microns or greater, 100 microns or greater, 120 microns or greater, 150 microns or greater, 175 microns or greater, 200 microns or greater, 250 microns or greater, 300 microns or greater, 350 microns or greater, 400 microns or greater, or 500 microns or greater). In some embodiments, the mixing channel has a height of 500 microns or less (for example, less than 500 microns, 450 microns or less, 400 microns or less, less than 400 microns, 350 microns or less, 300 microns or less, less than 300 microns, 275 microns or less, 250 microns or less, 200 microns or less, 150 microns or less, 100 microns or less, or 50 microns or less). In some embodiments, the mixing channel has a height of from 50 microns to 500 microns (for example, from 100 microns to 500 microns, from 200 microns to 500 microns, from 100 microns to 350 microns, or from 200 microns to 500 microns).

The mixing channel can have a width of at least 50 microns (for example, 75 microns or greater, 100 microns or greater, 120 microns or greater, 150 microns or greater, 175 microns or

greater, 200 microns or greater, 250 microns or greater, 300 microns or greater, 350 microns or greater, 400 microns or greater, or 500 microns or greater). In some embodiments, the mixing channel has a width of 500 microns or less (for example, less than 500 microns, 450 microns or less, 400 microns or less, less than 400 microns, 350 microns or less, 300 microns or less, less than 300 microns, 275 microns or less, 250 microns or less, 200 microns or less, 150 microns or less, 100 microns or less, or 50 microns or less). In some embodiments, the mixing channel has a width of from 50 microns to 500 microns (for example, from 100 microns to 500 microns, from 200 microns to 500 microns, from 100 microns to 350 microns, or from 200 microns to 500 microns).

The ratio of the culture chamber width to the to the largest cross-sectional dimension of the mixing channel is at least 2:1, at least 3:1, at least 4:1, at least 5:1, at least 6:1, at least 7:1, at least 8:1, at least 9:1, at least 10:1, at least 12:1, at least 15:1, at least 18:1, at least 20:1, at least 25:1, at least 50:1, at least 75:1, at least 100:1, at least 150:1, at least 200:1, at least 250:1, at least 300:1, at least 350:1, at least 400:1, at least 450:1, or at least 500:1. In some embodiments, the ratio of the culture chamber width to the largest cross-sectional dimension of the mixing channel is from 5:1 to 500:1, from 5:1 to 200:1, from 5:1 to 100:1, from 2:1 to 25:1, from 5:1 to 20:1, from 5:1 to 15:1, from 6:1 to 25:1, from 6:1 to 20:1, from 6:1 to 12:1, from 8:1 to 25:1, or from 10:1 to 25:1. A ratio of the culture chamber width to the largest cross-sectional dimension of the mixing channel which is greater than one (e.g., 2:1) defines a narrowing of channel width at the mixing channel inlet.

One or more channels in the microfluidic device can, in some embodiments, comprise a fluid mixing mechanism which facilitates the mixing of fluids flowing through the device. A fluid mixing mechanism induces turbulent flow so as to mix flowing fluids. Suitable mixing mechanisms include a serpentine or tortuous channel, a channel protrusion or indentation, a channel curvature, among other known mechanisms.

In some embodiments, a fluid mixing mechanism can be present in the mixing channel and/or in an isolation channel of the microfluidic device. For example, the microfluidic device can include an isolation channel that fluidly connects to the mixing channel to an isolation outlet. The isolation channel can form a part of the mixing channel or can be separate. In some embodiments, the isolation channel defines a path for fluid flow from the mixing channel to an isolation outlet. The isolation channel can comprise a serpentine geometry which enhances mixing as the fluids combine. Referring to Fig. 4, an isolation channel (230) having a serpentine geometry can be positioned within the device at a location advantageous for fluid mixing, for example, between a mixing intersection and a collection chamber. In some embodiments, the isolation channel is

positioned immediately adjacent a mixing intersection (e.g., immediately downstream).

The isolation channel can have a similar or narrowed width as compared to the mixing channel width. In some embodiments, the isolation channel can have a narrowed width as compared to the mixing channel width by at least several means. For example, the isolation channel can comprise one or more channel constriction domains (see, for example, the channel constriction domains (232) which form the narrowed width in the isolation channel in Fig. 4) disposed within the isolation channel between the isolation channel inlet and the isolation channel outlet. The channel constriction domains can produce a local vortex flow profile of fluid flowing in the device. In some embodiments, the isolation channel comprises at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten channel constriction domains. Any one or more channel constriction domains can be a protrusion and/or indentation in the channel sidewalls. The protrusion and/or indentation can have any shape, for example rounded, linear, triangular, irregular, etc. The protrusion and/or indentation can encircle the inner walls of the channel (e.g., as a ring), or one or more protrusions and/or indentation can be positioned on one or more inner sidewalls of the channel. Inclusion of a channel constriction domain can increase fluid flow turbulence and fluid mixing, where desirable.

Devices disclosed herein can include a collection chamber (240) fluidly connected to the isolation outlet (234). In some embodiments, the device can further comprise a magnet operatively coupled to the collection chamber to produce a magnetic field within the collection chamber. The magnet can be any magnet capable of providing a magnetic field within the collection chamber. In some embodiments, the magnetic field can include an oscillating magnetic field. An oscillating magnetic field is a magnetic field which varies regularly (e.g., automated periodic regularity) or irregularly (e.g., by user-based controls) over time. An oscillating magnetic field includes dynamic changes in the spatial orientation of the north and south magnetic poles, such that the direction of the magnetic field changes over time. Such changes can be cyclical or irregular. Inclusion of an oscillating magnet capable of providing an oscillating magnetic field within the collection chamber can induce magnetic probes (e.g., magnetic beads or particles) within the collection chamber to dynamically move inside the collection chamber along a direction of the magnetic field. As the direction of the magnetic field changes, the directional movement of magnetic particles within the collection chamber also changes. This can be used to foster interaction (and association/binding) between the magnetic particles and targets present in a fluid in the collection chamber. In some embodiments, the magnetic field can be obtained from a permanent magnet. The permanent magnet can be removed when not in use, for example, to switch off the magnetic field.

In some embodiments, the magnet can have any shape such as a toroidal shape. In some embodiments, the magnet comprises a Helmholtz coil or a permanent magnet.

The magnetic field can be present over the entire width of the collection chamber. In some embodiments, the magnetic field can be over a portion of the collection chamber, for example over at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% of the entire width of the collection chamber. Further, the magnetic field can be over other portions of the microfluidic device. For example, the magnetic field can be over one or more of a portion of or the entirety of the mixing channel, the mixing intersection, the particle inlet channel, or other components.

In some embodiments, the device can comprise a pump operably coupled to the microfluidic device. The pump can be any pump known in the art capable of inducing fluid flow within the device. In some embodiments, the pump can impart negative pressure within the device, thereby pulling fluid through a channel. Examples of suitable pumps can be found in US20170065978 and US20170001197, each of which are incorporated by reference in their entireties.

Turning now to Fig. 6, also disclosed is a microfluidic device (2000) comprising a cell culture chamber (2100) comprising a cell culture inlet (2102) and a cell culture outlet (2104), a fluid inlet channel (2202) and a particle inlet channel (2204), wherein the cell culture outlet (2104), the fluid inlet channel (2202), and the particle inlet channel (2204) fluidly converge at a mixing intersection (2200); a mixing channel (2300) fluidly connected to the mixing intersection (2200) and defining a path for fluid flow from the mixing intersection to a mixing outlet, wherein the ratio of a width of the cell culture chamber (2100) to the largest cross-sectional dimension of the mixing channel (2300) is at least 5:1; and a collection chamber (2400) fluidly connected to the mixing outlet and comprises a magnet operatively coupled to the collection chamber (2400) to produce a magnetic field within the collection chamber. The mixing channel (2300) can comprise an isolation channel (2302) disposed between the mixing intersection (2200) and the mixing outlet.

Another aspect of the microfluidic devices provided herein relates to multiplexed microfluidic devices which contain two or more sets of chambers and/or channels including the cell culture chamber, the mixing channel, the isolation channel, and the collection chamber. Configuring two or more of such channels and/or chambers on a single microfluidic device can increase sample-processing throughput and/or allow for parallel processing of at least two samples or portions of the sample for different fractions or manipulations. Two or more chambers and/or channels can be arranged in series, in parallel or in a combination thereof.

In some embodiments of a parallel multiplexed microfluidic device, two or more mixing channels can have separated sample inlets disposed on the same microfluidic device. Such arrangement can be employed for multiple fluid samples. Alternatively, the plurality of the mixing channels can be connected to the same sample inlets for parallel processing of the same fluid sample. Additionally, the two or more mixing channels can have separated outlets disposed on the same microfluidic device or be connected to the same outlet. In one or more embodiments, multiplexed microfluidic devices are contemplated having as many as 96 sample inlets.

The microfluidic devices of the present disclosure can be used in combination with the various compositions, devices, methods, products, and applications disclosed herein. In some embodiments, the microfluidic devices can be a stand-alone microfluidic device. In some embodiments, one or more microfluidic devices can be integrated as part of an equipment, a module or a system. In other embodiments, one or more microfluidic devices can be fluidically coupled to an equipment, a module or a system.

By way of example only, one or more microfluidic device and/or multiplexed microfluidic devices can be fluidically coupled to a detection module. As used herein, the term "fluidically coupled" refers to two or more devices and/or modules connected in an appropriate manner such that a fluid can pass or flow from one device or module to the other device or module. When two or more devices and/or modules are fluidically coupled together, additional devices and/or modules can be present between the two or more devices and/or modules.

Alternatively, two the two or more devices and/or modules can be connected such that a fluid can pass or flow directly from a first device or module to a second device or module without any intervening devices or modules. Two or more devices or modules can be fluidically coupled, for example, by connecting an outlet of a first device or module to an inlet of a second device or module using tubing, a conduit, a channel, piping or any combinations thereof.

The detection module can perform any method of detection disclosed herein or other methods known in the art. In some embodiments, the detection module can include a sample-treatment module before the sample is detected for analysis. For example, the exosomes (including or excluding the immunomagnetic particles) can be subjected to immunostaining before detection by microscopy. Examples of the detection module can include, without limitations, a microscope (e.g., a brightfield microscope, a fluorescence microscope, or a confocal microscope), a spectrophotometer (e.g., UV-Vis spectrophotometer), a cell counter, a biocavity laser (see, e.g., Gourley et al., J. Phys. D: Appl. Phys. 36: R228-R239 (2003)), a mass spectrometer, an imaging system, an affinity column, a particle sorter, e.g., a fluorescent activated cell sorter, capillary

electrophoresis, a sample storage device, and sample preparation device. In some embodiments, a computer system can be connected to the detection module, e.g., to facilitate the process of sample treatment, detection and/or analysis.

Methods of Making

The devices described herein can be made of any material that is compatible with a fluid sample. In some embodiments, the material for fabrication of the devices described herein can be penetrated by a magnetic field. In some embodiments, the material for fabrication of the devices described herein can be substantially transparent so that the sample therein can be photocleaved *in situ* or it can be viewed under a microscope, e.g., for *in situ* analysis of the magnetically-labeled exosomes. Exemplary materials that can be used to fabricate the microfluidic devices described herein can include, but are not limited to, glass, co-polymer, polymer or any combinations thereof. Exemplary polymers include, but are not limited to, polyurethanes, rubber, molded plastic, polymethylmethacrylate (PMMA), polycarbonate, polytetrafluoroethylene (TEFLON™), polyvinylchloride (PVC), polydimethylsiloxane (PDMS), polysulfone, and ether-based, aliphatic polyurethane.

The methods used in fabrication of any embodiments of the microfluidic devices described herein can vary with the materials used, and include 3D printing methods, soft lithography methods, microassembly, bulk micromachining methods, surface micro-machining methods, standard lithographic methods, wet etching, reactive ion etching, plasma etching, stereolithography and laser chemical three-dimensional writing methods, solid-object printing, machining, modular assembly methods, replica molding methods, injection molding methods, hot molding methods, laser ablation methods, combinations of methods, and other methods known in the art.

In specific embodiments, the microfluidic devices described herein can be fabricated using a 3D printer. For example, methods of making the microfluidic devices can include providing three pieces of PDMS molds including a base, wall, and top magnet holder as shown in FIG. 7. The mold can be printed out by a 3D printer. The molds can be coated with Sportline palladium at a thickness of 20 nm followed by assembly using methods known in the art. The PDMS cell chamber can be sized so that when it is filled, the cell culture chamber has an open end for chamber plug. PDMS can be cast by a 10:1 ratio with a linker reagent and incubated at a temperature of 40°C for 6 hours. After the PDMS is cured, it can be peeled out easily. Chip inlets and outlet can be punched by using puncher. The PDMS chips can then be post-bond on a hot pad at the

temperature of 40°C for 5 mins. The chips can be cleaned using DI water, and sterilized by autoclave (at 121°C for 30 mins).

Methods of Use

As discussed herein, the microfluidic devices disclosed can be used for isolating, capturing, engineering, and releasing engineered extracellular vesicles and various vesicle-like biological structures. In certain embodiments, the engineered extracellular vesicles are immunogenic exosomes. As used herein, the term "exosome" generally refers to externally released vesicles originating from the endosomic compartment or any cells, e.g., tumor cells (e.g., prostate cancer cells), and immune cells (e.g., antigen presenting cells, such as dendritic cells, macrophages, mast cells, T lymphocytes or B lymphocytes). Exosomes are generally membrane vesicles with a size of about 20-150 nm that are released from a variety of different cell types including tumor cells, red blood cells, platelets, lymphocytes, and dendritic cells. Exosomes can be formed by invagination and budding from the membrane of late endosomes. They can accumulate in cytosolic multivesicular bodies (MVBs) from where they can be released by fusion with the plasma membrane. Without wishing to be bound by theory, the process of vesicle shedding is particularly active in proliferating cells, such as cancer cells, where the release can occur continuously. When released from tumor cells, exosomes can promote invasion and migration. Thus, in some embodiments, the immunomagnetic particles described herein can be used to capture exosomes originating from cancer cells. Depending on the cellular origin, exosomes can recruit various cellular proteins that can be different from the plasma membrane including MHC molecules, tetraspanins, adhesion molecules and metalloproteinases. Among many subtypes of exosomes, the immunogenic exosomes with an intrinsic payload of MHC class I and II molecules and other co-stimulatory molecules are able to mediate immune responses, which opens up opportunities for the development of novel cancer vaccines and delivery in immunotherapy.

Accordingly, also provided herein are methods of producing immunogenic exosomes in a microfluidic device disclosed herein. The methods of producing immunogenic exosome complexes can comprise introducing cells into the cell culture chamber of the microfluidic device. The cells in the cell culture chamber can include any cells from which extracellular vesicles can be obtained. Such cells include dendritic cells, stem cells, immune cells, megakaryocyte progenitor cells, macrophages, or combinations thereof.

The method for producing immunogenic exosomes can further include culturing the cells under conditions allowing release of exosomes. In some embodiments, the methods can include

enriching or expanding the number of exosomes present in the cell sample through mediating their parent cell growth using stimulations known in the art. Conventional methods for culturing a parent cell to produce exosomes are known in the art and can be used in the methods disclosed herein. In some embodiments, the cells can be cultured for a period of time, e.g., at least about 30 mins, at least about 45 mins, at least about 1 hour, at least about 2 hours, at least about 3 hour, at least about 5 hours, at least about 6 hours, at least about 8 hours, at least about 10 hours, at least about 12 hours, at least about 15 hours, at least about 18 hours, at least about 20 hours, at least about 24 hours, at least about 30 hours, at least about 36 hours, at least about 40 hours, or at least about 48 hours.

The method for producing immunogenic exosomes complex can further comprise mixing the cell culture comprising exosomes with immunomagnetic particles for capturing the exosomes and a wash solution to form a mixture. In some embodiments, the methods include introducing the exosomes from the cell culture into a mixing channel and mixing the exosomes with immunomagnetic particles and a wash buffer to form a mixture. The immunomagnetic particles can be introduced into the mixing channel via the particle inlet channel and the wash buffer can be introduced into the mixing channel via the fluid inlet channel.

The immunomagnetic particles can selectively bind to the exosomes present in the cell culture to form exosome-bound immunomagnetic particles. Accordingly, the method can include allowing the exosomes to react with the immunomagnetic particles. The immunomagnetic particles can include a magnetic particle and be of any shape, including but not limited to spherical, rod, elliptical, cylindrical, and disc. In some embodiments, magnetic particles having a substantially spherical shape and defined surface chemistry can be used to minimize chemical agglutination and non-specific binding. As used herein, the term “magnetic particles” can refer to a nano- or micro-scale particle that is attracted or repelled by a magnetic field gradient or has a non-zero magnetic susceptibility. The magnetic particles can be ferromagnetic, paramagnetic or super-paramagnetic. In some embodiments, magnetic particles can be super-paramagnetic.

The magnetic particles can range in size from 1 nm to 5 microns. For example, magnetic particles can be about 500 nm to about 5 microns in size. In some embodiments, magnetic particles can be about 1 micron to about 5 microns in size. In some embodiments, magnetic particles can be about 1 micron to about 3 microns in size. Magnetic particles are a class of particles which can be manipulated using magnetic field and/or magnetic field gradient. Such particles commonly consist of magnetic elements such as iron, nickel and cobalt and their oxide compounds. Magnetic particles (including nanoparticles or microparticles) are well-known and methods for their

preparation have been described in the art. Magnetic particles are also widely and commercially available. A particularly preferred particle is a magnetic particle having a graphene-oxide layer or coating which is comprised of graphene-oxide nanosheets, as described in US 2018/0100853, filed October 9, 2017, incorporated by reference in its entirety herein.

The magnetic particles can be coated with a plurality of linkers comprising respective affinity probes (molecules) for capturing the target (such as an antigen peptide or antigenic epitope thereof) having no adverse effect on the magnetic property. In this regard, the magnetic particle can be functionalized with an organic moiety or functional group and photocleavable linker that can connect the magnetic particle to respective affinity probes for capturing the exosomes. Such organic moiety or functional groups can typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as amino groups, carboxylic acid groups, epoxy groups, tosyl groups, silica-like groups, carbonyl groups, amide groups, SO, SO_2, SO_2NH, SS, or a chain of atoms.

In certain embodiments, the magnetic particles can be coated with one member of an affinity binding pair that can facilitate the conjugation of the magnetic particles to the affinity probe for capturing the exosomes. The term "affinity binding pair" or "binding pair" refers to first and second molecules that specifically bind to each other. One member of the binding pair is conjugated with first part to be linked while the second member is conjugated with the second part to be linked. Exemplary binding pairs include any haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof (e.g., digoxigenin and anti-digoxigenin; mouse immunoglobulin and goat antimouse immunoglobulin) and nonimmunological binding pairs (e.g., biotin-avidin, biotin-streptavidin, biotin-neutravidin, hormone [e.g., thyroxine and cortisol-hormone binding protein, receptor-receptor agonist, receptor-receptor antagonist (e.g., acetylcholine receptor- acetylcholine or an analog thereof), IgG-protein A, IgG-protein G, IgG-synthesized protein AG, lectin-carbohydrate, enzyme- enzyme cofactor, enzyme-enzyme inhibitor, and complementary oligonucleotide pairs capable of forming nucleic acid duplexes), and the like. The binding pair can also include a first molecule which is negatively charged and a second molecule which is positively charged.

One example of using binding pair conjugation is the biotin-avidin, biotin-streptavidin or biotin-neutravidin conjugation. Accordingly, in some embodiments, the magnetic particles can be coated with avidin-like molecules (e.g., streptavidin or neutravidin), which can be conjugated to biotinylated linkages for use as capturing molecules.

In some embodiments, the magnetic particles can be further functionalized with a cleavable chemical moiety that can link the magnetic particles to the affinity probe for capturing the

exosomes, and is susceptible to an externally-applied cleavage agent/conditions, e.g., UV light, pH, redox potential or the presence of degradative molecules such as enzymes. In specific examples, the cleavable linker can be conjugated to a member of a binding pair (such as biotin) at one functional end to link to the magnetic particles, and the other functional end provides an affinity probe for capturing exosomes. Thus, after the exosomes bound magnetic particles are separated from a fluid sample, the exosomes can be separated from the magnetic particles, if needed, by cleaving the cleavable chemical moiety between the magnetic particles and the affinity probe.

Exemplary cleavable linking groups include, but are not limited to, photocleavable and redox cleavable linking groups (e.g., -OC(O)NH-, -S-S-, and -$C(R)_2$-S-S-, wherein R is H or C_1-C_6 alkyl); phosphate-based cleavable linking groups (e.g., -O-P(O)(OR)-O-, -O-P(S)(OR)-O-, and -O-P(S)(H)-S-, wherein R is optionally substituted linear or branched C_1-C_{10} alkyl); acid cleavable linking groups (e.g., hydrazones, esters, and esters of amino acids, -C=NN- and - OC(O)-); ester-based cleavable linking groups (e.g., -C(O)O-); peptide-based cleavable linking groups, (e.g., linking groups that are cleaved by enzymes such as peptidases and proteases in cells, e.g., -$NHCHR_AC(O)NHCHR_BC(O)$-, where R_A and R_B are the R groups of the two adjacent amino acids).

In some embodiments, the cleavable linking group is a photocleavable group that can be cleaved by UV light. Specific examples of photocleavable groups include ortho nitrobenzyl derivatives and benzylsulfonyl such as 6-nitroveratryloxycarbonyl (NVOC), 2-nitrobenzyloxycarbonyl (NBOC), α,α-dimethyl-dimethoxybenzyloxycarbonyl (DDZ), ortho-nitrobenzyl (ONB), 1-(2-nitrophenyl)ethyl (NPE), alpha-carboxy-2-nitrobenzyl (CNB), 4,5-dimethoxy-2-nitrobenzyl (DMNB), l-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE), 5-carboxymethoxy-2-nitrobenzyl (CMNB), and (5- carboxymethoxy-2-nitrobenzyl)oxy)carbonyl (CMNCBZ). It will be appreciated that the substituents on the aromatic core are selected to tailor the wavelength of absorption, with electron donating groups (e.g., methoxy) generally leading to longer wavelength absorption. For example, nitrobenzyl (NB) and nitrophenylethyl (NPE) are modified by addition of two methoxy residues into 4,5-dimethoxy-2-nitrobenzyl and 1 -(4,5-dimethoxy-2-nitrophenyl)ethyl, respectively, thereby increasing the absorption wavelength range to 340-360 nm. Additional examples of the photoremovable protecting groups include multiply substituted nitro aromatic compounds containing a benzylic hydrogen ortho to the nitro group, wherein the substituent may include alkoxy, alkyl, halo, aryl, alkenyl, nitro, halo, or hydrogen. Other materials which may be used include o-hydroxy-α-methyl cinnamoyl derivatives,

photocleavable groups based on the coumarin system, such as BHC (such as described in U.S. Patent No. 6,472,541, the disclosure of which is incorporated by reference herein), photocleavable group comprising the pHP group (such as described in Givens et al., J. Am. Chem. Soc. 122 2687-2697 (2000), the disclosure of which is incorporated by reference herein), ketoprofen derived linkers, other ortho-nitro aromatic core scaffolds include those that trap nitroso byproducts in a hetero Diels Alder reaction (generally discussed in U.S. Patent Application No. 2010/0105120, the disclosure of which is incorporated by reference herein), nitrodibenzofurane (NDBF) chromophore, or a diazo-azide. Further examples of photocleavable groups may be found in, for example, Patchornik, J. Am. Chem. Soc. (1970) 92:6333 and Amit et al., J. Org. Chem. (1974) 39:192, the disclosures of which are incorporated by reference herein.

As discussed above, a photocleavable group is one whose covalent attachment to a molecule (such as to a member of a binding pair example biotin at one functional end and the other functional end to an affinity probe) is cleaved by exposure to light of an appropriate wavelength. In one aspect, release of the affinity probe and/or binding pair occurs when the conjugate is subjected to ultraviolet light or near ultraviolet light. For example, photorelease of the affinity probe may occur at a wavelength ranging from about 200 to 380 nm (the exact wavelength or wavelength range will depend on the specific photocleavable group used, and could be, for example, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, or 380 or some range therebetween). In another aspect, release of the affinity probe may occur when the conjugate is subjected to visible light. For example, photorelease of the affinity probe may occur at a wavelength ranging from about 380 to 780 nm (the exact wavelength or wavelength range will depend on the specific photocleavable group used, and could be, for example, 380, 400, 450, 500, 550, 600, 650, 700, 750, or 780, or some range therebetween).

As described herein, the magnetic particles further comprise an affinity probe (also referred to herein as a molecule for capturing the exosomes or capturing molecule). As used herein, the term “affinity probe” or “capturing molecule” refers to any molecule, cell or particulate material. Suitable affinity probes comprising a magnetic particle are described in US20170065978 and US20170001197, each of which are incorporated by reference in their entireties. The affinity probes can comprise a binding element which specifically binds the target (exosome or other extracellular vesicles) of interest. For example, the binding element can be a nucleic acid oligomer, antibody, enzyme, hormone, growth factor, cytokine (e.g., inflammatory cytokines), proteins, peptide, prion, lectin, oligonucleotide, carbohydrate, lipid, molecular and chemical toxin or other binding element which has high affinity and high specificity for the target, and specificity for a

designated surface protein on the target. One or more binding elements (e.g., a peptide) can be attached to the magnetic particle via the cleavable linker by methods known in the art. Generally, a binding element has an affinity constant (Ka) greater than about 10^5 M^{-1} (e.g., 10^6 M^{-1}, 10^7 M^{-1}, 10^8 M^{-1}, 10^9 M^{-1}, 10^{10} M^{-1}, 10^{11} M^{-1}, and 10^{12} M^{-1} or more) with the target, particularly exosome or other extracellular vesicles .

In certain embodiments, the affinity probe includes an antigenic peptide or antigenic epitope thereof. As used herein, the term "antigens" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to elicit the production of antibodies capable of binding to an epitope of that antigen. An antigen may have one or more epitopes. The term "antigen" can also refer to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen," as used herein, also encompasses T-cell epitopes. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. An antigen can have one or more epitopes (B- and T-epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

As described above, the affinity probe (antigen) can be a protein or a peptide. In some embodiments, the protein or peptide can be essentially any protein that can activate immune cells and/or prime immune-responses, bind to a rare cell, e.g., a circulating tumor cell, a stem cell and/or a microbe. By way of example only, if the target species is cancer, exemplary proteins or peptides or other molecule that can be used to generate cancer-affinity probes can include, but are not limited to, MAGE-A3, gp-100, HER-2, p53, PSA-1, or MART-1, EGFR, ERCC1, CXCR4, EpCAM, CEA, ErbB-2, E-cadherin, mucin-1, cytokeratin, PSA, PSMA, RRM1, androgen receptor, estrogen receptor, progesterone receptor, IGF1, cMET, EML4, or leukocyte associated receptor (LAR).

In some embodiments, the affinity probe can be an antibody or a portion thereof, or an antibody-like molecule. In some embodiments, the capturing molecule can be an antibody or a portion thereof, or an antibody-like molecule that is specific for detection of a rare-cell, e.g., a circulating tumor cell, a stem cell and/or a microbe biomarker. In some embodiments, the affinity probe can be an aptamer. In some embodiments, the affinity probe can be a DNA or RNA aptamer. In some

embodiments, the affinity probe can be a cell surface receptor ligand. Exemplary, cell surface receptor ligand includes, for example, a cell surface receptor binding peptide, a cell surface receptor binding glycopeptide, a cell surface receptor binding protein, a cell surface receptor binding glycoprotein, a cell surface receptor binding organic compound, and a cell surface receptor binding drug. Additional cell surface receptor ligands include, but are not limited to, cytokines, growth factors, hormones, antibodies, and angiogenic factors. In some embodiments, any art-recognized cell surface receptor ligand that can bind to a rare cell, e.g., a circulating tumor cell, a stem cell and/or a microbe, can be used as an affinity probe on the magnetic particles described herein. In one or more embodiments, an affinity probe is selected to target an immunostimulatory molecule presented on the surface of the target (e.g., exosome), such as MHC class I molecule, an MHC class II molecule, an interleukin, TNFα, IFNγ, RANTES, G-CSF, M-CSF, IFNα, CTAPIII, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1β and combinations thereof. More preferably, upon binding of the affinity probe (and subsequent release of the target), the resulting engineered target comprising the bound affinity probe enhances the immunogenic potential of the released target. For example, the binding of an MHC class I surface protein by the affinity probe creates a complex that will (in therapeutic use) enhance recognition and uptake of the engineered target by an antigen presenting cell for stimulation and activation of the immune system. The peptides are preferably 15 amino acid residues or less in length, more preferably 13 residues or less in length, even more preferably 12 residues or even 11 residues or less in length.

Example affinity probes include those listed in the Table below:

	SEQ ID NO:		SEQ ID NO:
Peptide 1: SIINFEKL	1	Peptide 2: RSV M2 82-90 peptide sequence SYIGSINNI	2
Peptide 3: Fusion 85-93 peptide sequence KYKNAVTEL	3	Peptide 4: M187-195 peptide sequence NAITNAKII	4
MAGE-A1 161-169: EADPTGHSY	5	MAGE-A3 168-176: EVDPIGHLY	6
MAGEA-10 254-262: GLYDGMEHL	7	MAGEA3 112-120: KVAELVHFL	8
MAGEA1 278-286: KVLEYVIKV	9	MAGEA3 271-279: FLWGPRALV	10

MAGEA3 112-120 (alternative version): KVAEELVHFL	11	MAGEA2 157-166: YLQLVFGIEV	12
MAGE-A4 230–239: GVYDGREHTV	13	MAGE-C1 1083-1091: KVVEFLAML	14
MAGE-C2 191-200: LLFGLALIEV	15	MAGE-C2 336-344: ALKDVEERV	16
MAGEA3 97-105: TFPDLESEF	17	MAGEA5 5-12: HNTQYCNL	18
Prostate Specific Antigen 146-154: KLQCVDLHV	19	Carcinogenic Embryonic Antigen (CEA) 694-702: GVTYACFVSNL	20
Carcinogenic Embryonic Antigen (CEA) 652-660: TYACFVSNL	21	G250 (renal cell carcinoma) 217-225: HLSTAFARV	22
HER-2/neu 435-443: ILHNGAYSL	23	HER-2/neu 63-71: TYLPTNASL	24
HER-2 434-443: ILHDGAYSL	25	Neu/Her-2/Erbb2 proto-oncoprotein 66-74: TYVPANASL	26
gp100 (pmel17) 209-217: IMDQVPFSV	27	gp100-intron 4 (170-178): VYFFLPDHL	28
gp100 (pmel17) 154-162: KTWGQYWQV	29	gp100 (pmel17) 476-485: VLYRYGSFSV	30
gp100 (pmel) 209-217: ITDQVPFSV	31	gp100 (pmel) 280-288 (288V): YLEPGPVTV	32
gp100: YLEPGPVTA	33	gp100 (pmel17) 25-33: KVPRNQDWL	34
gp100 (pmel17) 17-25: ALLAVGATK	35	gp100-intron 4 (170-178): VYFFLPDHL	36
HER-2/neu 369-377: KIFGSLAFL	37	p53 264-272: LLGRNSFEV	38
p53 187-197: GLAPPQHLIRV	39	p53 149-157: SLPPPGTRV	40
p53 139-147: KLCPVQLWV	41	p53 65-73: RMPEAAPPV	42
p53 103-111: YLGSYGFRL	43	Prostatic Acid Phosphatase-3 (PAP-3): FLGYLILGV	44
PSM P2 (prostate): ALFDIESKV	45	Prostate Stem Cell Antigen (PSCA) 14-22: ALQPGTALL	46
MelanA / MART 26-35: ELAGIGILTV	47	Prostate Specific Antigen-1 (PSA-1) 141-150: FLTPKKLQCV	48

MUC-1 12-20: LLLLTVLTV	49	Human Mena protein (overexpressed in breast cancer): GLMEEMSAL	50
HER-2/neu 689-697: RLLQETELV	51	HER-2/neu (85–94): LIAHNQVRQV	52
Prostate Specific Antigen-1 (PSA-1) 154-163: VISNDVCAQV	53	Prostate Specific Antigen-1 153-161: CYASGWGSI	54
PSA 65-73: HCIRNKSVI	55	EGF-R-479 350-359: KLFGTSGQKT	56
EGF-R 1138-1147: YLNTVQPTCV	57	VEGFR2 400-408: VILTNPISM	58
VEGFR2/KDR fragment 1 614-624: FSNSTNDILI	59		

It will be appreciated that the foregoing peptides, suitable for affinity probes, are also exemplary of active agents or moieties for surface loading onto the engineered target.

The immunomagnetic particles (that is magnetic particles bound to the affinity probe) are preferably formed before the mixing process. For example, the affinity probe (e.g., an antigenic peptide bound to biotin via a cleavable linker) and streptavidin coated magnetic particles are mixed together for an effective period of time for the biotinylated affinity probe linkages to substantially completely coat the entire surface of the avidin coated particles.

Thus, the affinity probe is preferably added to the streptavidin coated magnetic particles for a period of time, before adding the exosome containing cell culture. In such embodiments, the affinity probe can be first added to the streptavidin coated magnetic particles for a period of time sufficient for at least a portion of the added amount of affinity probe to bind with the streptavidin coated magnetic particles (and preferably for complete binding of affinity probe so as to coat the entire surface of the particle with probe linkages extending therefrom).

The exosomes present in the exosome containing cell culture are then added into the same fluid sample, where the exosomes can bind to the affinity probe, which have already formed a conjugate with the streptavidin coated magnetic particles.

In some embodiments, the immunomagnetic particles can be separately formed before being introduced into the mixing channel of the device.

The amount of the immunomagnetic particles required to be added into the sample can depend on a number of factors, including, but are not limited to, volume of the sample to be processed, valency of the magnetic particles available for conjugation with the affinity probe, expected abundance of the exosomes present, and any combinations thereof. Too high amounts of

the immunomagnetic particles added into the device can induce non-specific binding and/or clogging inside the microfluidic device. Too low amounts of the immunomagnetic particles can result in a low capture efficiency. One skilled in the art can determine the concentration of the immunomagnetic particles and capturing molecules.

The exosomes can be allowed to mix with the immunomagnetic particles for any period of time, e.g., seconds, minutes or hours. In some embodiments, the exosomes can be mixed with the immunomagnetic particles for at least about 1 min, at least about 2 mins, at least about 5 mins, at least about 10 mins, at least about 15 mins, at least about 30 mins, at least about 1 hour, at least about 2 hours or more. A person having ordinary skill in the art can readily determine an optimum time for mixing time, based on a number of factors, including, but not limited to, the affinity of the immunomagnetic particles with the exosomes, concentrations, mixing temperature and/or mixing speed. However, in one or more embodiments, exosomes are mixed with the particles for 1 hour or less.

The exosomes and immunomagnetic particles can be introduced into the sample inlets of the microfluidic device at any flow rate that provides a sufficient residence time for the mixture to retain in the mixing channel and isolation channel of the microfluidic device described herein. In some embodiments, the samples can be introduced at a flow rate of between 0.1 uL/min to 1 uL/min. The sample fluids can be introduced into the inlet of the microfluidic device by any methods known to a skilled artisan. For example, a flow generator can be connected to at least one of the inlets and the outlet of the microfluidic device described herein. Non-limiting examples of a flow generator can include a peristaltic pump, a syringe pump and any art-recognized pump that can be generally used to flow a fluid through the microfluidic device.

The method of producing immunogenic engineered targets can further include capturing the exosomes bound to the immunomagnetic particles by applying a magnetic field within a collection or engineering chamber. In some embodiments, the magnet has a strong magnetic field strength sufficient to create a magnetic field gradient to cause the magnetically-labeled exosomes to separate from the fluid sample in the collection chamber. The immobilized magnetically-labeled exosomes can be removed from the microfluidic device for further processing. Preferably, the captured exosomes are further engineered and loaded with additional active moieties on the surface or internally as discussed herein. Subsequently, the method includes photolytically cleaving the exosomes bound to the immunomagnetic particles for releasing intact exosomes coated with active moieties or loaded internally with active agents.

The released target (exosomes) can be provided as a pharmaceutical composition. The

pharmaceutical composition can include the immunogenic exosomes and a pharmaceutically acceptable excipient. It will be appreciated that the active moieties can be tailored to provide either a specific adaptive immune response against a target condition, or can be selected more generally to activate the innate immune system against a variety of infections or conditions.

The methods described herein can be used to process samples in real time. For example, the methods allow real-time, continuous harvesting and antigenic modification of exosomes with subsequent photo-release downstream on-demand.

As described herein the methods can be used to produce an immunogenic exosome complex or other immunogenic vesicle-like structures. In certain embodiments, the immunogenic exosome complex can comprise an antigen peptide conjugated to a surface of an exosome. The methods described herein for making the immunogenic exosome complex provides complexes with a significantly higher activation rate for T-cells than non-engineered exosomes. In some examples, the immunogenic exosome complex can activate T-cell by at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% compared to a native exosome. Because of the improved activation rate, the immunogenic complexes described herein can be used in cancer immunotherapy.

Accordingly, methods of treating disease in a subject using the immunogenic complex are disclosed. The method can include administering to the subject a composition comprising an immunogenic complex. In some embodiments, the disease can be an infection. In some examples, the disease can be cancer. The method can further comprise administering a chemotherapeutic agent that has been loaded into the target.

Additional advantages of the various embodiments of the invention will be apparent to those skilled in the art upon review of the disclosure herein and the working examples below. It will be appreciated that the various embodiments described herein are not necessarily mutually exclusive unless otherwise indicated herein. For example, a feature described or depicted in one embodiment may also be included in other embodiments, but is not necessarily included. Thus, the present invention encompasses a variety of combinations and/or integrations of the specific embodiments described herein.

As used herein, the phrase "and/or," when used in a list of two or more items, means that any one of the listed items can be employed by itself or any combination of two or more of the listed items can be employed. For example, if a composition is described as containing or excluding components A, B, and/or C, the composition can contain or exclude A alone; B alone;

C alone; A and B in combination; A and C in combination; B and C in combination; or A, B, and C in combination.

The present description also uses numerical ranges to quantify certain parameters relating to various embodiments of the invention. It should be understood that when numerical ranges are provided, such ranges are to be construed as providing literal support for claim limitations that only recite the lower value of the range as well as claim limitations that only recite the upper value of the range. For example, a disclosed numerical range of about 10 to about 100 provides literal support for a claim reciting "greater than about 10" (with no upper bounds) and a claim reciting "less than about 100" (with no lower bounds).

The devices, systems, and methods of the appended claims are not limited in scope by the specific devices, systems, and methods described herein, which are intended as illustrations of a few aspects of the claims. Any devices, systems, and methods that are functionally equivalent are intended to fall within the scope of the claims. Various modifications of the devices, systems, and methods in addition to those shown and described herein are intended to fall within the scope of the appended claims. Further, while only certain representative devices, systems, and method steps disclosed herein are specifically described, other combinations of the devices, systems, and method steps also are intended to fall within the scope of the appended claims, even if not specifically recited. Thus, a combination of steps, elements, components, or constituents may be explicitly mentioned herein or less, however, other combinations of steps, elements, components, and constituents are included, even though not explicitly stated.

The term “comprising” and variations thereof as used herein is used synonymously with the term “including” and variations thereof and are open, non-limiting terms. Although the terms “comprising” and “including” have been used herein to describe various embodiments, the terms “consisting essentially of” and “consisting of” can be used in place of “comprising” and “including” to provide for more specific embodiments of the invention and are also disclosed. Other than where noted, all numbers expressing geometries, dimensions, and so forth used in the specification and claims are to be understood at the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, to be construed in light of the number of significant digits and ordinary rounding approaches.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

By way of non-limiting illustration, examples of certain embodiments of the present disclosure are given below.

EXAMPLES

The following examples set forth methods in accordance with the invention. It is to be understood, however, that these examples are provided by way of illustration and nothing therein should be taken as a limitation upon the overall scope of the invention.

Example 1: Microfluidic On-demand Engineering of Exosomes towards Cancer Immunotherapy

Abstract: Extracellular nanovesicles (≤1 μm), particularly exosomes (30-150 nm), are emerging delivery system in mediating cellular communications, which have been observed for priming immune responses by presenting parent cell signaling proteins or tumor antigens to immune cells. In this example, a streamlined microfluidic cell culture platform for harvesting, antigenic modification, and photo-release of surface engineered exosomes directly in one workflow is provided. The PDMS microfluidic cell culture platform is replicated from a 3D-printed mold. By engineering antigenic peptides on exosome surface (e.g., gp-100, MART-1, MEGA-A3), the effective antigen presentation and T cell activation can be achieved. This has been demonstrated by using the on-chip culture of human blood-derived leukocytes for engineering secreted exosomes in real-time with melanoma tumor peptides. gp100-specific CD8 T cells which were purified from the spleen of 2 Pmel1 transgenic mice was tested. Significantly higher T-cell activation level (~30%) induced by engineered exosomes was observed compared to non-engineered exosomes. This microfluidic platform serves as an automated and highly integrated cell culture device for rapid, and real-time production of therapeutic exosomes that could advance cancer immunotherapy.

METHODS AND MATERIALS

3D Printing and microfluidic device fabrication: three pieces of molds for PDMS chip fabrication, including a base, wall, and top magnet holder were provided. The mold was designed by using the SolidWorks® 2017 and printed out by the 3D printer of Project 1200 from 3D Systems. The multiple pieces had the finest structure in 50 μm, and with channel height at 50 μm. The cell culture chamber was designed with 1000 μm diameter, 500 μm height chamber. All molds were coated with Sportline palladium at the thickness of 20 nm. All three pieces were assembled using the PDMS chip. The PDMS was filled with a height under 500 μm, so the cell culture chamber left an open end for chamber plug. PDMS was cast by a 10:1 ratio with a linker reagent,

and incubated at the temperature of 40°C for 6 hours. After the PDMS cured, it could be peeled out easily. Chip inlets and outlet were punched by using 0.75 mm puncher. Piranha treated glass and PDMS were both high-voltage plasma for at least 30 seconds. The PDMS chips were then post-bond on the hot pad at the temperature of 40°C for 5 mins. The chips were cleaned by DI water, and sterilized by autoclave (at 121°C for 30 mins).

On-chip cell culture and exosome collection, engineering, and releasing: The cell cartridges (8 mm coverslip) were first cleaned with distilled water, and air dried inside the bio-hood. Then, they were autoclaved at 121°C for 30 mins. The cartridges were set in a 24-well plate, and 500 μL of 0.1 mg/mL poly-D-lysine hydrobromide (MP Biomedicals) was added to each well, and incubated at the room temperature for 5 mins. 1 mL of MD water was added to each well for 3 mins and repeated for two times to clean the cell cartridges, and then sit for air dried inside the bio-hood and stored for future use.

4 μL β2-microglobulin (Sigma-Aldrich) and 10 μL of each protein (gp100, MAGE-A3, and MART-1) were mixed with 186 μL 1× PBS to the modification solution at a final volume of 200 μL. The B-inlet was kept blocked and the modification solution was pumped from A-inlet and the washing buffer from C-inlet through the chip at the volume flow rate of 1 μL/min for 10 mins, and 0.1 μL/min for 10 mins, and static was set for another 10 mins. A washing step was processed from both A-inlet and C-inlet at the volume flow rate of 1 μL/min for 15 mins. The bottom side magnet was removed and the near UV turned on to treat the major chamber for 10 mins. Another washing step from A-inlet and C-inlet was applied at the volume flow rate of 1 μL/min for 20 mins, to collect the calved exosome from outlet about 20 μL.

Ultracentrifugation and exosomes staining: The collected 20 μL exosomes were added to the ultracentrifuge tube and diluted to the final volume of 1 mL for centrifugation (Thermo Scientific™ Sorvall™ MTX) under 1,500 rcf for 30 mins. The supernatant was removed and transferred to a fresh ultracentrifuge tube. The mixture was then processed at the speed of 100,000 rcf for 1 hour. Exosomes were stained by the PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane Labeling (Sigma-Aldrich). The staining solution was prepared with 2 μL of PKH67 and 1 mL of diluent C. Any remaining solution in the tube was discarded and 1 mL of Diluent C was added to re-suspend with gentle pipetting. The stained solution to the ultracentrifuge tube, pipette mixed, and reacted at the room temperature for 3.5 mins. 2 mL of FBS (exosome depleted) was added to quench the free dye. 1.5 mL of 0.971 M sucrose solution was added for density gradient centrifugation. Another 6.5 mL of complete media was added to raise the volume to 10 mL. The ultracentrifuge was set at 100,000 rcf for 1 hour. The supernatant was discarded

and the dye ring washed carefully without reaching the center of the ring. Another 2 mL of 1X PBS was added to re-suspend the pellet. The ultracentrifuge at the speed of 100,000 rcf was ran for another 1 hour. The supernatant was sucked away, and another 100 µL of 1X PBS added to re-suspend the pellet. All steps were kept under sterile condition, and 1 µL of Penicillin-Streptomycin (ATCC®, Catalog# 30-2300, Lot#63525409) was added to the collected exosome, to inhibit and kill bacteria remaining in the solution. The collected exosomes were stored at 4°C for less than 1 week and stored at -20°C for up to one month.

Exosome uptake: THP-1 cells (ATCC®, TIB-202™) was cultured by using ATCC-formulated RPMI-1640 Medium (ATCC®, Catalog# 30-2001, Lot# 64331683) plus 10 % exosome-depleted FBS for the completed media. The monocytes cells were sub-cultured at the number of 8*105/mL, and by using the alternative media changing method. The cells were used for exosomes up taking experiment at the density of $5*10^5$/mL. 200 uL of the monocytes cells were transferred to the 48-well plate with totally 11 wells. 20 uL of normal exosome (NE) was added to 5 wells, also 20 µL of engineered exosome (EE) to another 5 wells, and one well left as a negative control. Time intervals were set at 0 hours, 0.5 hours, 1 hour, 2 hours, 3 hours, and 4 hours. At each time section, 100 uL of cell suspension media was removed from the cytocentrifuge, at the speed of 400 RPM for 4 mins. Glass slides were collected and 100 uL of Fixative Solution (ThermoFisher®, Catalog# R37814, Lot# 17B285301) was added to cells' spot. The mixture incubated at room temperature for 18 mins, and then the solution removed. 100 µL of 1× PBS buffer was added to the cells' spot, and left to set at room temperature for 3 mins. 1× PBS buffer was removed and the cells' spot gently washed by the distilled water. The slide was dried without droplet remains on the slide, and 50 µL of 500 nM DAPI (ThermoFisher®, Catalog# D1306, Lot# 1844202) applied to the cells' spot, covered from light, and incubated at room temperature for 4 mins. The DAPI solution was then quickly removed and followed with a sufficient amount of 1× PBS buffer twice with 2 mins for each time. The cells' spot was washed with distilled water, and briefly dried without droplet remain on the slide. One drop of ProLong™ Gold Antifade Mountant (ThermoFisher®, Ref# P10144, Lot1887458) was applied and the slide covered with 25x25 #1.5 coverslip without any trapped bubble. The slide was stored at room temperature for 24 hours before imaging under a confocal microscope.

RESULTS

3D-printing molded microfluidic cell culture device for on-inline harvesting exosomes: A facile and low-cost approach for making a PDMS-based on-chip cell culture microfluidic device using a 3D-printed mold has been developed. The culture chip contains an on-chip cell culture

chamber with 1 mm diameter and 0.5 mm height for on-chip growing cells and collecting exosomes derived from culture medium at downstream. The cell culture chamber is left open on top for applying a PDMS-made, finger-push plug for medium exchange and pushing the medium to downstream collection channels. The bottom of the cell culture chamber has an outlet channel about 200 μm wide and 200 μm high (B-Inlet) for introducing culture medium to mix with immunomagnetic isolation beads (A-Inlet). The C-Inlet is used to introduce washing buffer driven by a syringe pump. Fig. 4 demonstrates the mixing process through the A-Inlet and B-Inlet and exit to exosome isolation channel (serpentine channel) under the observation of the fluorescence microscope using a fluorescence dye solution. Fig. 5(b) records the immunomagnetic beads mixing within the serpentine channel. Human blood-derived leucocytes were cultured in the culture device with the morphology showing in Fig. 5(c). Few red blood cells were still observed as a cup shape. The secreted exosomes were isolated, captured, and photo-released from the outlet of the chip, and characterized by SEM imaging shown in Fig. 5(d).

A photo-cleavable linker was conjugated with bi-function of biotin and NHS chemistry on both ends. The biotin group anchors the photo-cleavable linker to the surface of streptavidin immunomagnetic beads, and the NHS group conjugates the MHC-I peptide via the primary amine, as shown in Fig. 7A. The MHC class I molecules are heterodimers that consist of two polypeptide chains, α, and β2-microglobulin. The two chains are linked noncovalently via interaction of b2m and the α3 domain. The other two domains α1 and α2 are folded to make up a groove for binding to 8-10 amino acid peptides (MHC-I binding peptide). The MHC-I/peptide binding complex will be displayed to cytotoxic T cells consequently for triggering an immediate response from the immune system. Once the MHC-I positive exosomes are captured by tumor targeting antigenic (TTA) peptide and retained by immunomagnetic beads within the capture chamber with the magnetic field, the antigenic loading buffer with saturated TTA peptides will be introduced via C-Inlet to completely bind and occupy the rest available MHC-I peptide binding sites. This antigenic surface engineering process can substantially enhance the loading amount of TTA peptides to captured MHC-I positive exosomes and boost the potency to activate T-cells.

Further characterized was the binding strength between MHC-I peptide modified photo-release immunomagnetic beads with MCH-I positives exosomes labeled with fluorescence as shown in Fig. 7B. The MHC-I antibody serves as the positive control to evaluate the binding strength between tumor targeting antigen peptides and MHC-I positive exosomes. Because of the stronger binding strength between MHC-I/peptide complex, it has a higher potential to activate T

cell anti-tumor responses. gp-100 was shown to have a stronger ability to form MHC-I/peptide complex and the binding strength is even stronger than MHC-I antibody (95% vs 84.8%).

The performance of on-demand photo-release was characterized in Figs. 8A-8E. With the comparison between positive control and negative control, the fluorescent-labeled exosomes were captured and released by measuring fluorescence intensity from beads aggregates under an invert fluorescence microscope. The SEM imaging approach was used to confirm the photo-release process. By comparing the SEM imaging of bead surface before and after photocleavage, there were no identifiable exosome particles presented on the surface of beads, indicating the good photo-release performance. The UV exposure time was characterized as well for reaching 98% photo-cleavage rate within 8-minute UV exposure. The size distribution of engineered exosomes and non-engineered exosomes was evaluated, which showed an appropriate size range of exosomes between 50 nm- 200 nm, confirming that engineered exosomes are maintaining good integrity.

The side-effect of UV exposure on exosome molecular contents was investigated, which shows non-detectable changes in terms of exosomal proteins, DNAs, and RNAs under 10-minute UV treatment (Fig. 13).

In order to evaluate the potency and integrity of engineered exosomes released from a microfluidic cell culture device via on-demand photo-release, the exosomes from a chip outlet was harvested and labeled with green fluorescence. gp-100 engineered exosomes and non-engineered exosomes was incubated with dendritic monocytes for monitoring cellular uptake with a one-hour interval. The cells were then fixed and the nuclei were stained with DAPI. The green dots shown in Fig. 9A are labeled exosomes, which are abundantly distributed around cellular nuclei. The cellular uptake begins within one hour and the uptake speed is much faster than the non-engineered exosomes. After 4 hours, it was observed that both engineered exosomes and non-engineered exosomes were cleared by the lysosome pathway. This observation indicated that gp-100 engineered exosomes are much more active for dendritic monocytes uptake. The expression of Cytokine IFN-ɤ was monitored from incubating gp-100 engineered exosome with dendritic monocytes using ELISA. Compared with the incubation of non-engineered exosomes, the IFN-ɤ expression level was much higher for 48 hours after continuously monitoring, with a nearly 2-fold increase. The gray dash line in the Fig. 9B indicates the positive control using PWM protein as the stimulator. The dendritic cellular morphology upon stimulation was shown in Fig. 14. Compared with negative control without stimulation, the both PWM protein and gp-100 engineered exosomes gave significant influence on changing to round floating dendritic cells. The gp-100 engineered

exosomes showed higher stimulation rate for Cytokine IFN-ɤ production than control PWM protein stimulation.

Further investigated was the potency of gp-100 engineered exosome for activating CD8+ T cells undergoing proliferation and cytolysis. It was observed that gp-100 engineered exosomes have the capacity to activate transgenic T cells in the presence of activated dendritic cells. The gp100-specific CD8 T cells were purified from the spleen of 2 Pmel1 transgenic mice by magnetic cell sorting and labeled with Cell Trace Violet proliferation dye. The purified T cells were cultured alone (T cells only) and mixed at a 3:1 ratio with naïve JAWS cells (an immature dendritic cell line derived from a C57BL/6 mouse), T cells + JAWS cells, or JAWS cells that were activated for 48 hours with 200 ng/mL (T cells + Activated JAWS cells). Engineered exosomes bearing the gp100 peptide were added to the T cell cultures at increasing ratios of exosomes: dendritic cells (25, 50 and 100). Fig. 10A. The cells and exosomes were co-cultured for 5 days and then CD8 T cells were analyzed by flow cytometry for Cell Trace Violet dilution as a measure of proliferation. With T cell only condition as the negative control, it was observed that the proliferation rate of CD8+ T cells cultured with gp-100 exosomes activated JAWS showed more than 30% increase, which indicated that gp-100 engineered exosomes has strong potency to activate T cell cytolysis. Fig. 10B. The developed microfluidic on-demand antigenic surface engineering and photo-release of exosomes could be a powerful tool for developing an effective exosome-based vaccine and delivery system for advancing Cancer Immunotherapy.

Immunogenic potency was also investigated for bovine respiratory syncytial virus (BRSV). T cells and activated JAWS cells were incubated with increasing concentrations of BRSV antimicrobial peptide-engineered exosomes (exosomes engineered with Peptide 4: M187-195 peptide NAITNAKII, SEQ ID NO:4). The immune-stimulation of CD8+ T cell proliferation is linearly responded to the dose of engineered exosomes which is more effective than using high dose peptide vaccines. The BRSV engineered exosomes have the capacity to activate BRSV M-specific T cells in the presence of activated dendritic cells. C57BL/6 mice were immunized twice subcutaneously with 20 nM BRSV M187-196 adjuvanted in QuilA. At least 4 weeks after the final immunization, the animals were euthanized and spleens were collected. CD8+ T cells and CD11c+ splenic dendritic cells were isolated by magnetic cell separation. CD8+ T cells were labeled with Cell Trace Violet proliferation dye. The purified T cells were cultured alone (T cells only), or were mixed at a 3:1 ratio with CD11c+ splenic DC (T cells + DC). DC cells were left unstimulated or were treated with 200 ng/mL LPS to induce DC activation. Engineered exosomes loaded with the BRSV peptide using the above-described microfluidic platform were added to the

T cell cultures at increasing ratios of exosomes: dendritic cells (25, 50 and 100). Negative control wells did not receive exosomes. Positive control wells were treated with 1 nM or 5 nM pure M187-196 peptide. The cells and exosomes were co-cultured for 5 days and then CD8 T cells were analyzed by flow cytometry for Cell Trace Violet dilution as a measure of proliferation. The results are shown in Fig. 11. All results support that the disclosed method for capture, antigenic loading, and photo-release can effectively produce antimicrobial peptide engineered exosomes which lead to successful activation of T cells with high potency.

Example 2: *In vivo* administration of engineered exosomes

The above immunogenic potency study used transgenic mice which were injected with engineered exosomes using proprietary disclosed method. The exosomes were engineered with gp-100 or BRSV M187-196 peptide on the surface using the above-described streamlined/continuous microfluidic process, and suspended in the PBS buffer for *in vivo* Intraperitoneal injection through tail. We observed no injection site reactions or adverse responses (injection site swelling, irritation, etc.) from the injected mice. Mice were observed twice per day for the first 72 hours after the injections and no adverse reactions were noted, indicating general *in vivo* safety of the engineered exosomes and related compositions.

CLAIMS:

1. A method for engineering a biological target in a microfluidic device comprising:
mixing in a microfluidic mixing channel of the microfluidic device a biological sample suspected of containing the biological target with a plurality of immunomagnetic particles in a fluid to form a mixture;
allowing the biological target, if present, to react and bind with the immunomagnetic particles in the mixture to form a particle/target complex;
immobilizing the particle/target complex in a chamber of the microfluidic device by applying a magnetic field within the chamber;
engineering the target in the particle/target complex by contacting said target with a plurality of active moieties or active agents, wherein the surface of said target is surface modified with said active moieties or wherein said plurality of active agents are loaded within said target to yield an engineered target; and
photolytically releasing said engineered target from said particle/target complex, wherein said particle remains immobilized in said chamber and wherein said engineered target is washed downstream to an outlet of said microfluidic device.

2. The method of claim 1, wherein said biological target is selected from the group consisting of cells, extracellular vesicles, and vesicle-like cell fractions.

3. The method of claim 2, wherein said extracellular vesicles are exosomes or microsomes.

4. The method of claim 3, said method further comprising:
introducing cells into a cell culture chamber of the microfluidic device;
culturing the cells under conditions allowing release of said exosomes; and
introducing the exosomes into said mixing channel for mixing with said plurality of immunomagnetic particles.

5. The method of claim 4, wherein the cells are selected from the group consisting of dendritic cells, stem cells, immune cells, megakaryocyte progenitor cells, and macrophages.

6. The method of claim 1, wherein said active moieties recognize and specifically bind to surface proteins on the biological target.

7. The method of claim 6, wherein substantially all of said surface proteins are bound with active moieties after said engineering step, such that said target is substantially coated with said active moieties.

8. The method of claim 1, wherein said immunomagnetic particles have a diameter of 500 nm or greater.

9. The method of claim 1, wherein each of said immunomagnetic particles reacts with a plurality of said biological targets in said sample, such that said particle/target complex comprises a plurality of said biological targets bound to a single, central particle.

10. The method of claim 9, wherein each particle binds a single type of biological target at a time, such that the same type of biological target is bound to each particle.

11. The method of claim 1, wherein said photolytically releasing comprises exposing said particle/target complex to light in said microfluidic chamber.

12. The method of claim 11, wherein said target is released within about 15 minutes after said exposing.

13. The method of claim 1, wherein the method is carried out in a continuous process in said microfluidic device, preferably within 90 minutes or less from said mixing to collection of said engineered target at said outlet.

14. The method of claim 1, wherein the active moieties are antigenic peptides.

15. The method of claim 1, wherein said active agents are drugs, nucleotides, CRISPR Cas9 systems, small molecule compounds, chemotherapeutics, and the like.

16. The method of claim 1, wherein said microfluidic device planar substrate comprising an inlet in fluid communication with said chamber via a microfluidic channel extending between the inlet and chamber, said chamber being downstream of said inlet and being in fluid communication with an outlet, said outlet being downstream of said chamber.

17. The method of claim 16, wherein said microfluidic device comprises a plurality of inlets for introducing said sample, said immunomagnetic particles, and wash buffer into said device.

18. The method of claim 16, wherein said microfluidic channel has a serpentine path of flow to facilitate mixing of said immunomagnetic particles and said sample.

19. The method of claim 16, said device further comprising a cell culture chamber in fluid communication with said microfluidic channel.

20. The method of any one of claims 1-19, wherein the immunomagnetic particles each comprise a plurality of photocleavable linkers extending from the particle surface, each with a respective affinity probe at the terminal end thereof, for capturing the biological target.

21. The method of claim 20, wherein the affinity probe comprises an antigenic peptide or antigenic epitope thereof.

22. The method of claim 21, wherein the affinity probe includes an antigenic peptide selected from MAGE-A3, gp-100, HER-2, p53, PSA-1, and MART-1.

23. The method of claim 20, wherein binding of said affinity probe with said biological target form an immunostimulatory complex, which upon release from the particle enhances antigen presentation and activation of immune cells and priming immune responses.

24. The method of claim 20, wherein the affinity probe is selected for specificity for an immunostimulatory molecule selected from the group consisting of MHC class I molecules, MHC class II molecules, interleukins, TNFα, IFNγ, RANTES, G-CSF, M-CSF, IFNα, CTAPIII, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1β, and combinations thereof.

25. The method of claim 20, wherein the photocleavable linker is conjugated with the particle surface via a biotin moiety.

26. The method of claim 20, wherein the affinity probes are from the same class or same type of compound as the active moieties.

27. A composition comprising an engineered biological target prepared by the method of claim 1.

28. The composition of claim 27, wherein said engineered biological target is an exosome, said exosome comprising a plurality of active moieties attached to the surface.

29. The composition of claim 28, wherein said active moieties are antigenic peptides or antigenic epitopes thereof.

30. The composition of claim 29, wherein said antigenic peptide selected from MAGE-A3, gp-100, HER-2, p53, PSA-1, and MART-1.

31. The composition of claim 29, wherein said antigenic peptide is selected for specificity for an immunostimulatory molecule selected from the group consisting of MHC class I molecules, MHC class II molecules, interleukins, TNFα, IFNγ, RANTES, G-CSF, M-CSF, IFNα, CTAPIII, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1β, and combinations thereof.

32. The composition of claim 31, wherein said antigenic peptide and said immunostimulatory molecule bind to form an immunostimulatory complex at the surface of said exosomes, which enhances antigen presentation and activation of immune cells and priming immune responses of said exosomes.

33. The composition of claim 27, wherein said engineered biological target is an exosome, said exosome comprising a plurality of active agents loaded therein.

34. The composition of claim 33, wherein said active agents are selected from the group consisting of nucleotides, drugs, chemotherapeutics, small molecule compounds, CRISPR Cas9 systems, and the like.

35. A method of activating immune cells and/or priming immune responses, said method comprising contacting an immune cell with a composition according to any one of claims 27-34.

36. The method of claim 35, wherein said contacting comprises administering said composition to a subject in need thereof.

37. The method of claim 36, said method effective for activating the innate or adaptive immune system of said subject against a condition.

38. The method of claim 37, wherein said condition is an infection.

39. The method of claim 37, wherein the condition is cancer.

40. The method of claim 39, further comprising administering a chemotherapeutic agent.

41. A microfluidic device for use in engineering a biological target, said device comprising:

a cell culture chamber dimensioned to maintain biological material in a three-dimensional configuration;

a mixing channel fluidly connected to the cell culture chamber and comprising a plurality of sample inlet channels disposed along the mixing channel, wherein the ratio of a width of the cell culture chamber to the largest cross-sectional dimension of the mixing channel is at least 5:1;

an isolation channel defining a path for fluid flow from the mixing channel to an isolation outlet; and

a collection chamber fluidly connected to the isolation outlet and comprises a magnet operatively coupled to the collection chamber to produce a magnetic field within the collection chamber.

42. A microfluidic device for use in engineering a biological target, said device comprising:

a cell culture chamber comprising a cell culture inlet and a cell culture outlet,

a fluid inlet channel and a particle inlet channel, wherein the cell culture outlet, the fluid inlet channel, and the particle inlet channel fluidly converge at a mixing intersection;

a mixing channel fluidly connected to the mixing intersection and defining a path for fluid flow from the mixing intersection to a mixing outlet, wherein the ratio of a width of the cell culture chamber to the largest cross-sectional dimension of the mixing channel is at least 5:1; and

a collection chamber fluidly connected to the mixing outlet and comprises a magnet operatively coupled to the collection chamber to produce a magnetic field within the collection chamber.

43. The microfluidic device of claim 42, wherein the mixing channel comprises an isolation channel disposed between the mixing intersection and the mixing outlet.

44. The microfluidic device of any one of claims 41-43, wherein the isolation channel has a serpentine geometry.

45. The microfluidic device of any one of claims 41-44, wherein the isolation channel comprises a channel constriction domain that decreases in width.

46. The microfluidic device of claim 45, wherein the isolation channel comprises a plurality of channel constriction domains for producing local vortex flow profile, preferably at least 5 channel constriction domains.

47. The microfluidic device of any one of claims 41-46, wherein the ratio of the cell culture chamber width to the largest cross-sectional dimension of the mixing channel is from 5:1 to 500:1, from 5:1 to 20:1, or from 6:1 to 12:1.

48. The microfluidic device of any one of claims 41-47, wherein the cell culture chamber has a volume of about 200 microliters or greater, preferably from about 200 microliters to about 1 milliliter.

49. The microfluidic device of any one of claims 41-48, wherein the mixing channel has a height and a width, wherein each of the height and the width is at least 50 microns, preferably between 50 and 500 microns.

50. The microfluidic device of any one of claims 41-49, further comprising a pump operably coupled to the microfluidic device.

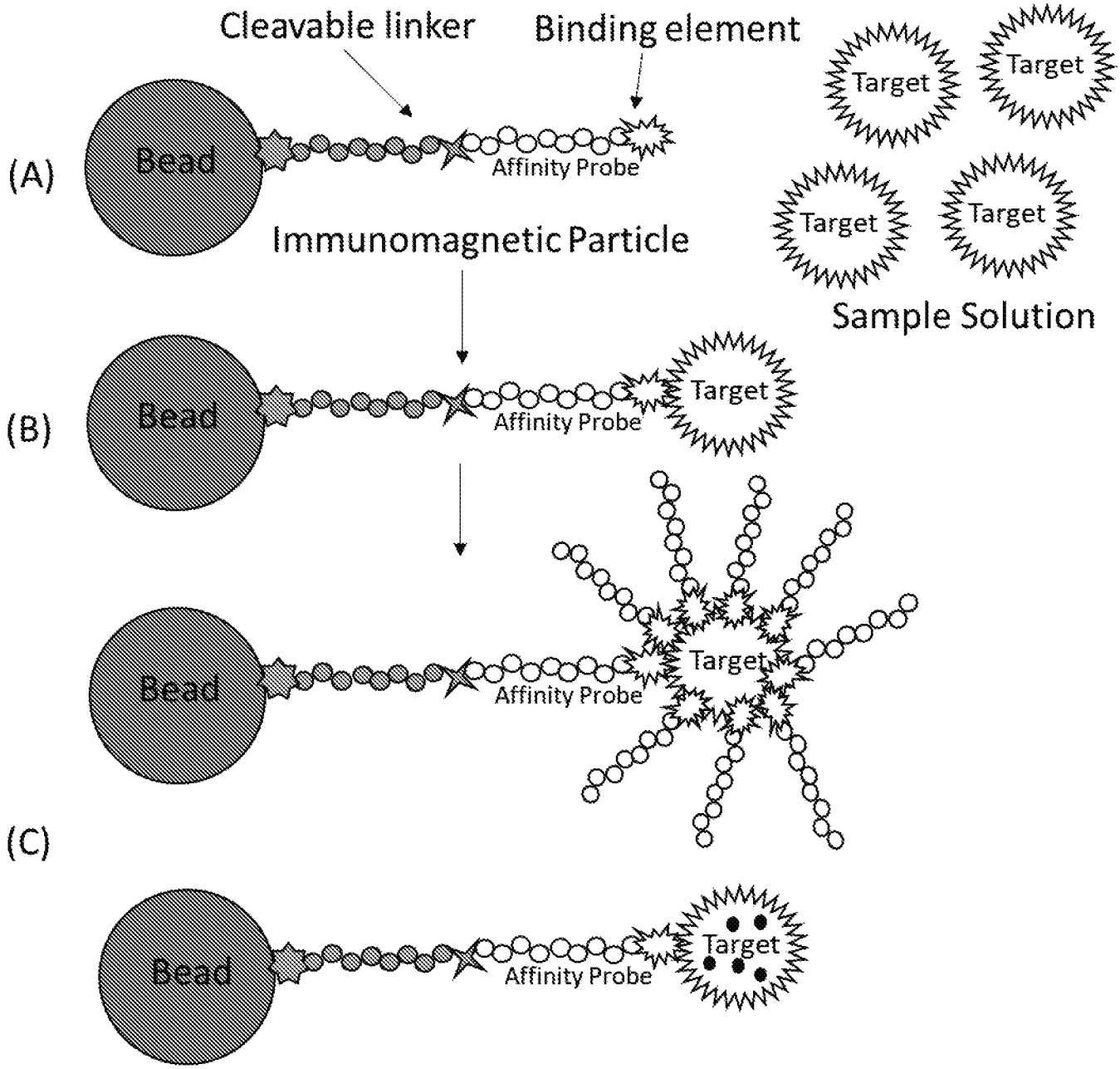

Cleavable linker
Binding element
Target
Target
Target
Target
(A)
Bead
Affinity Probe
Immunomagnetic Particle
Sample Solution
(B)
Bead
Affinity Probe
Target
Bead
Affinity Probe
Target
(C)
Bead
Affinity Probe
Target

FIG. 1

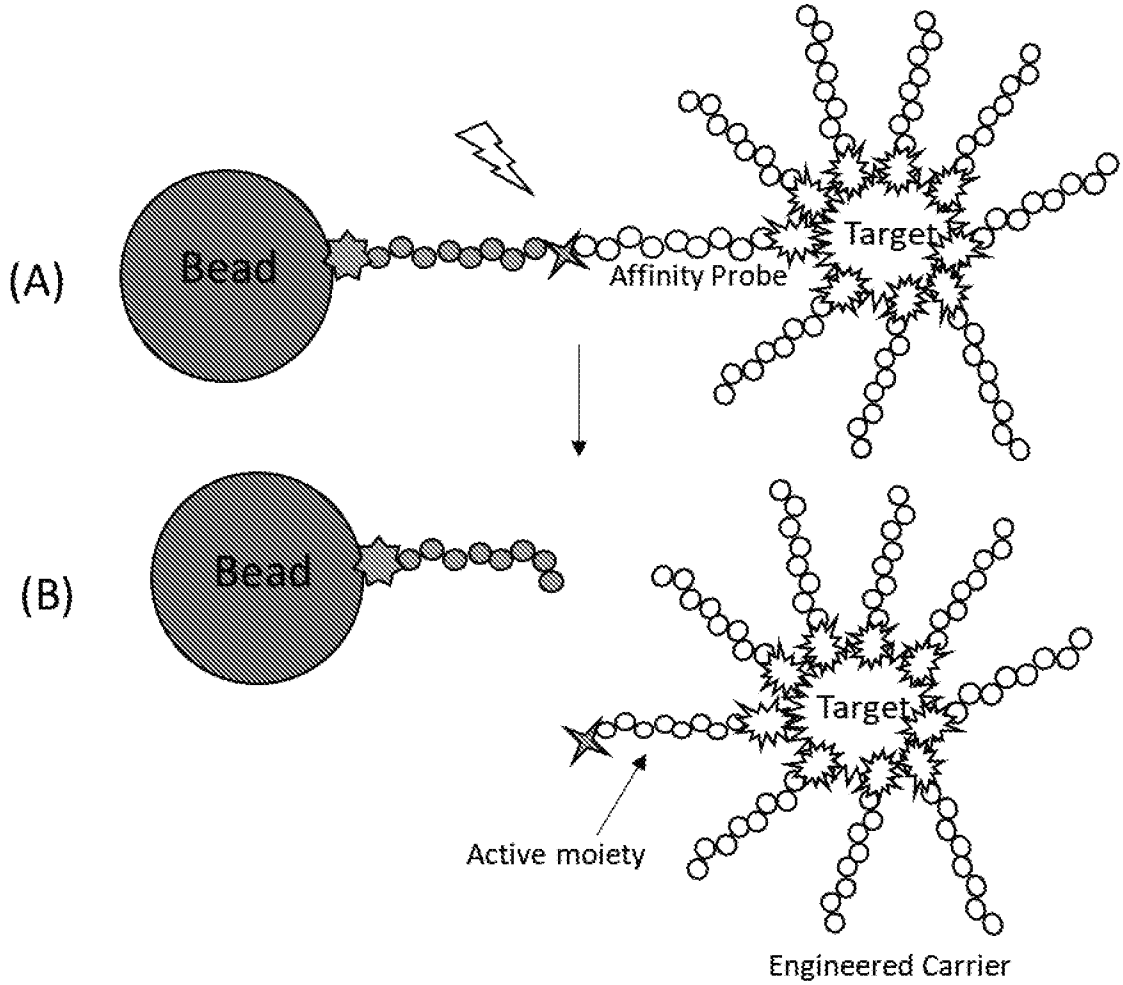

(A)
Bead
Affinity Probe
Target
(B)
Bead
Target
Active moiety
Engineered Carrier

FIG. 2A

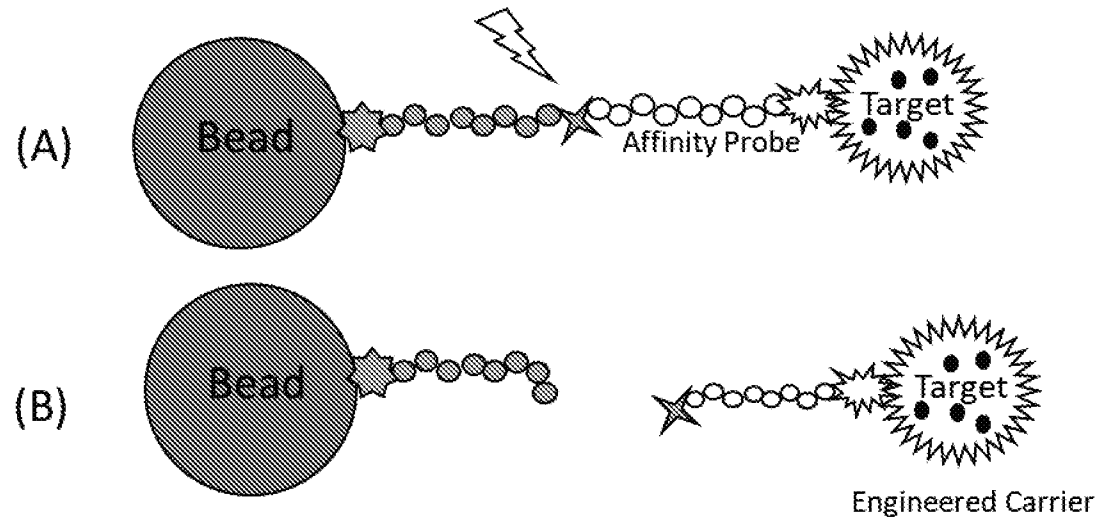

(A)
Bead
Affinity Probe
Target
(B)
Bead
Target
Engineered Carrier

FIG. 2B

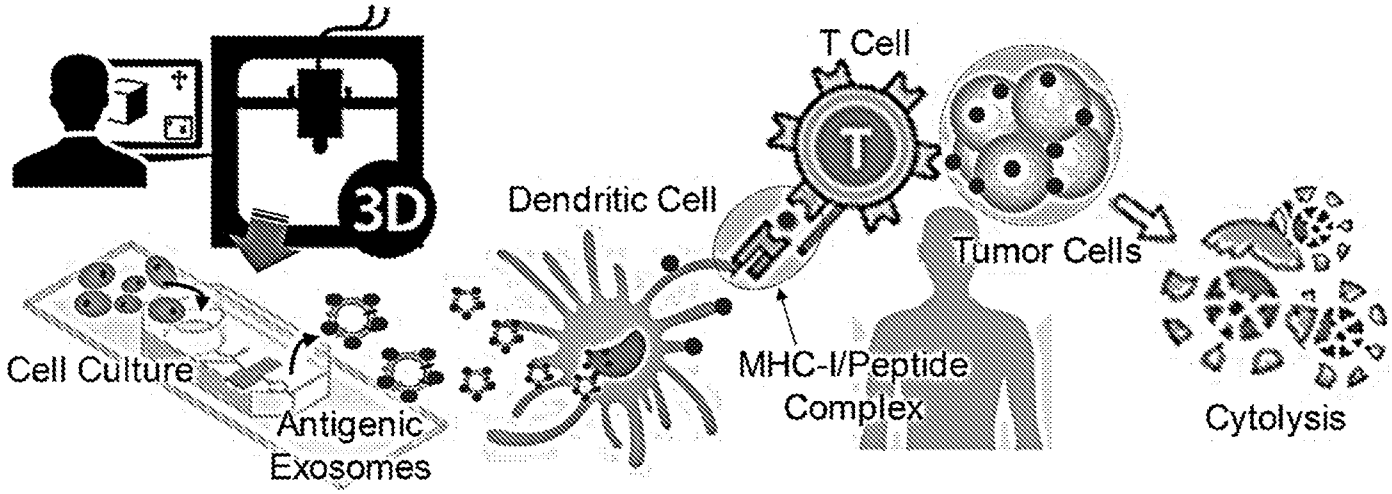

3D
T Cell
Dendritic Cell
Tumor Cells
Cell Culture
Antigenic Exosomes
MHC-I/Peptide Complex
Cytolysis

FIG. 3

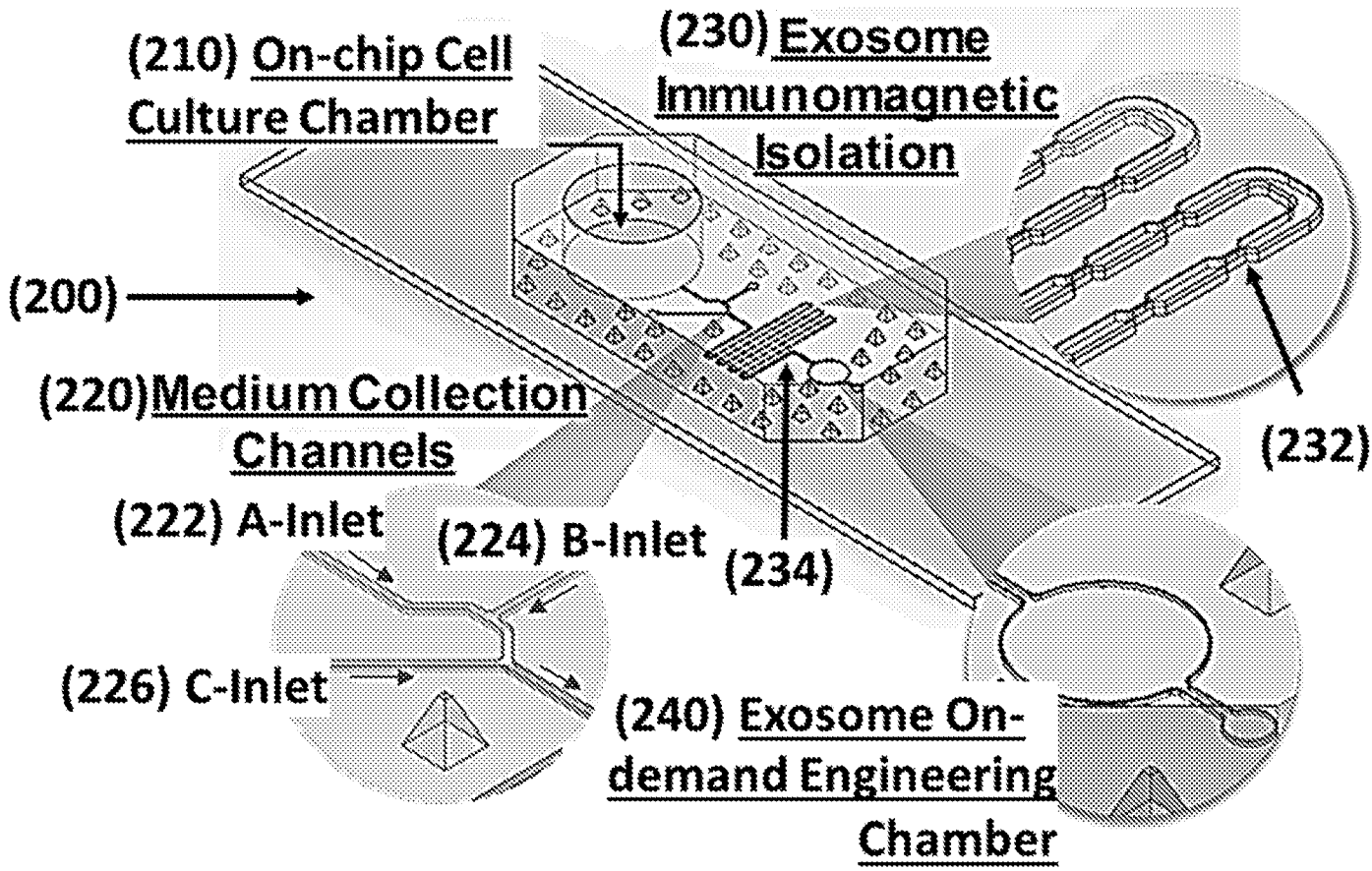

(210) On-chip Cell Culture Chamber
(230) Exosome Immunomagnetic Isolation
(200)
(220) Medium Collection Channels
(232)
(222) A-Inlet
(224) B-Inlet
(234)
(226) C-Inlet
(240) Exosome On-demand Engineering Chamber

FIG. 4

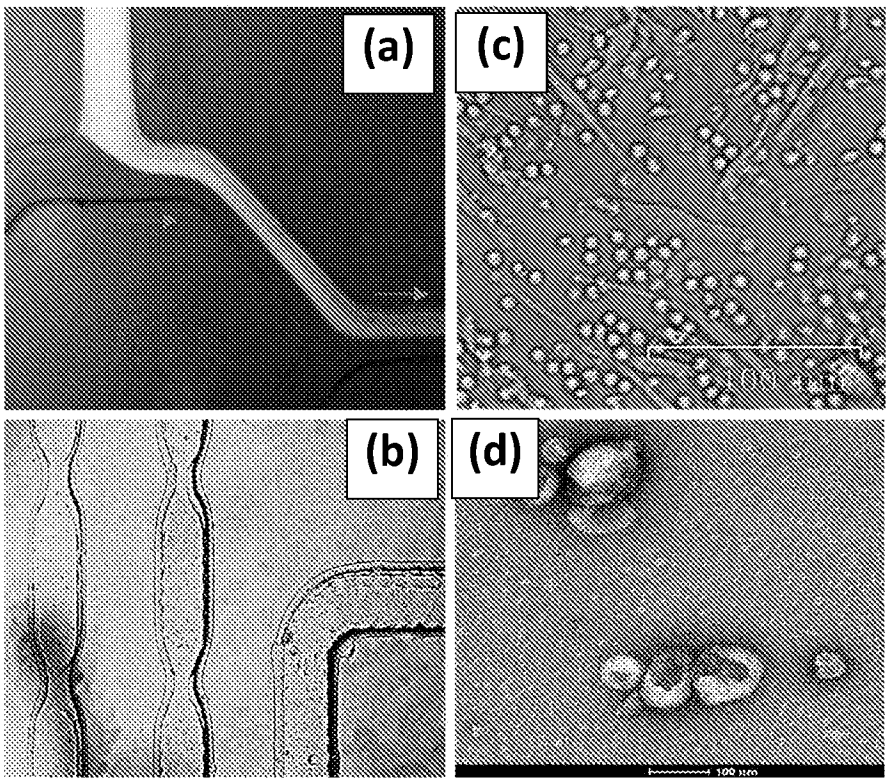


FIG. 5

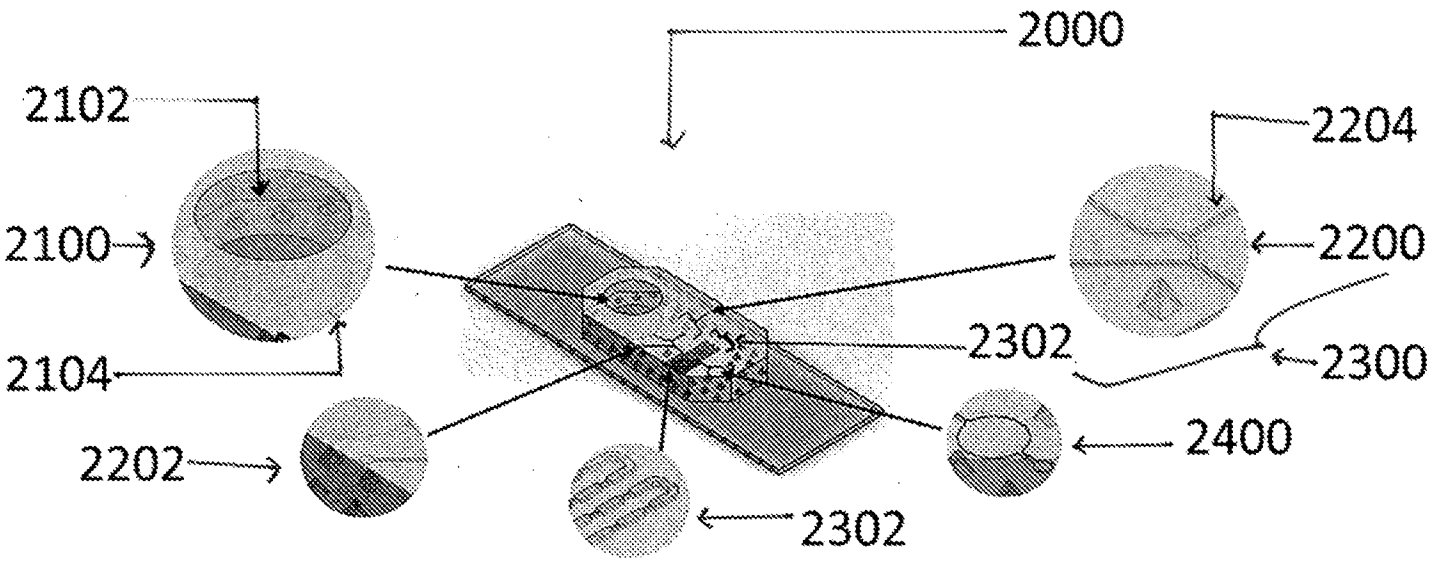


FIG. 6

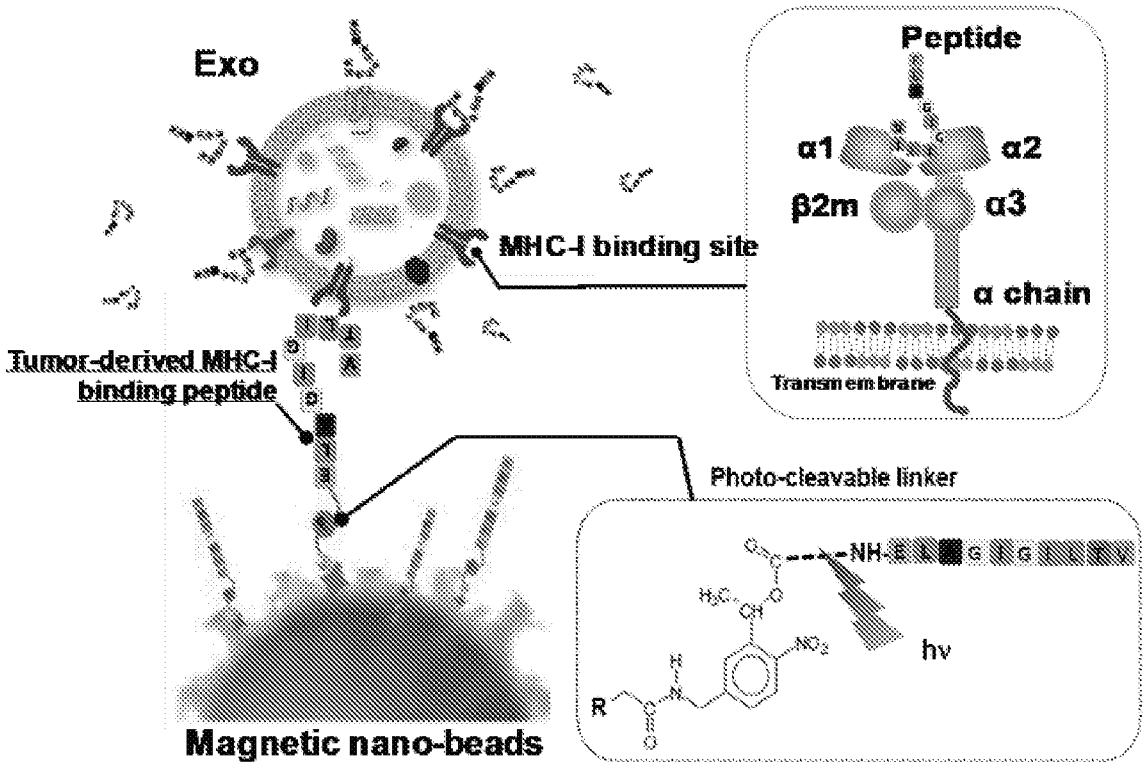

Exo
Peptide
α1
α2
β2m
α3
α chain
Transmembrane
MHC-I binding site
Tumor-derived MHC-I binding peptide
Photo-cleavable linker
hv
Magnetic nano-beads

FIG. 7A

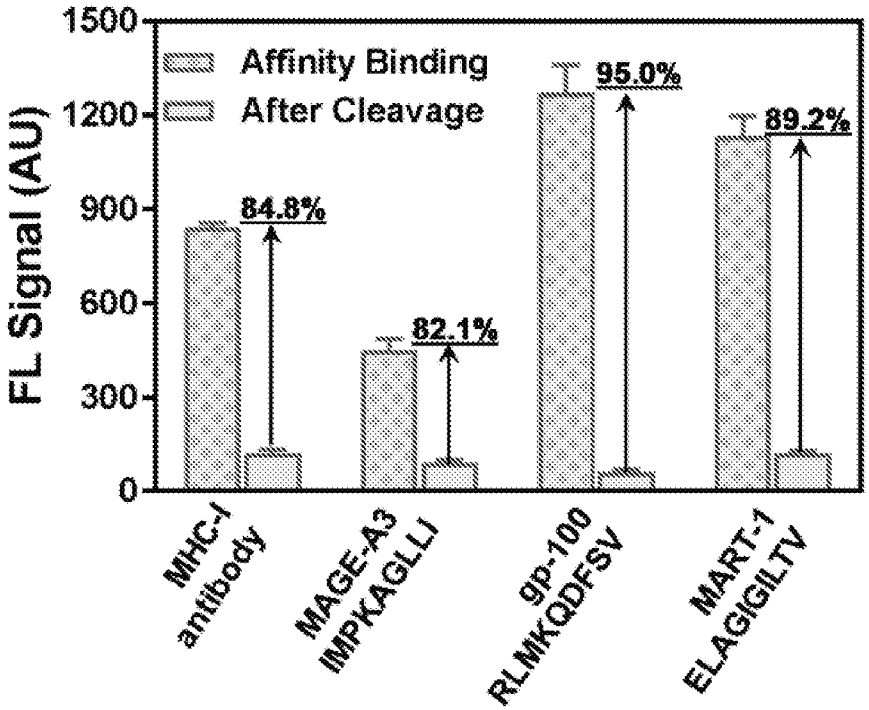

1500
1200
900
600
300
0
FL Signal (AU)
Affinity Binding
After Cleavage
84.8%
82.1%
95.0%
89.2%
MHC-I antibody
MAGE-A3 IMPKAGLLI
gp-100 RLMKQDFSV
MART-1 ELAGIGILTV

FIG. 7B

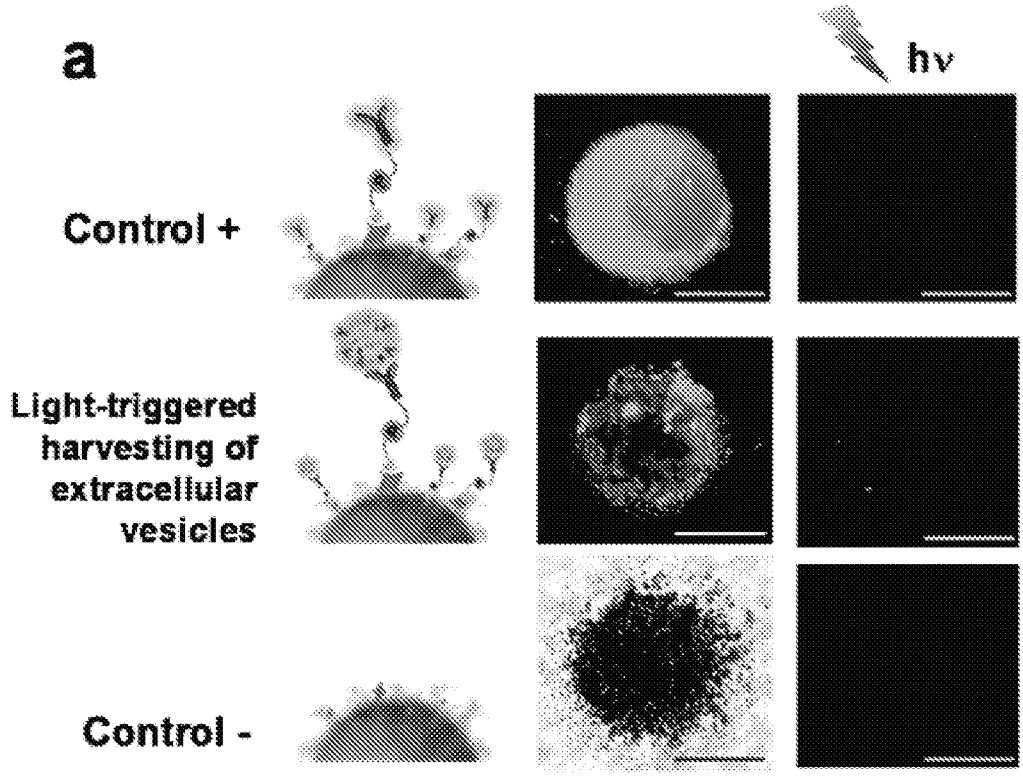

a
hν
Control +
Light-triggered harvesting of extracellular vesicles
Control -

FIG. 8A

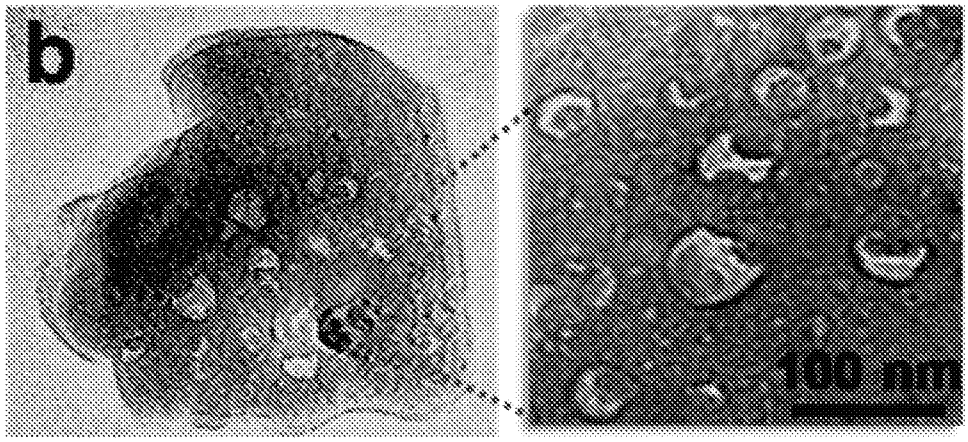

b
100 nm

FIG. 8B

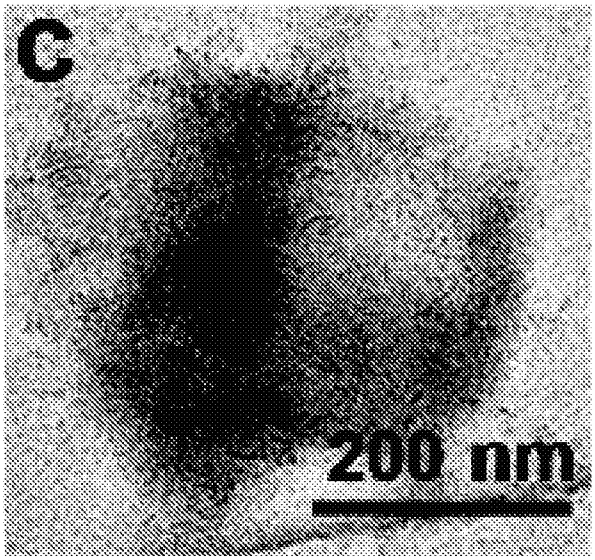

c
200 nm

FIG. 8C

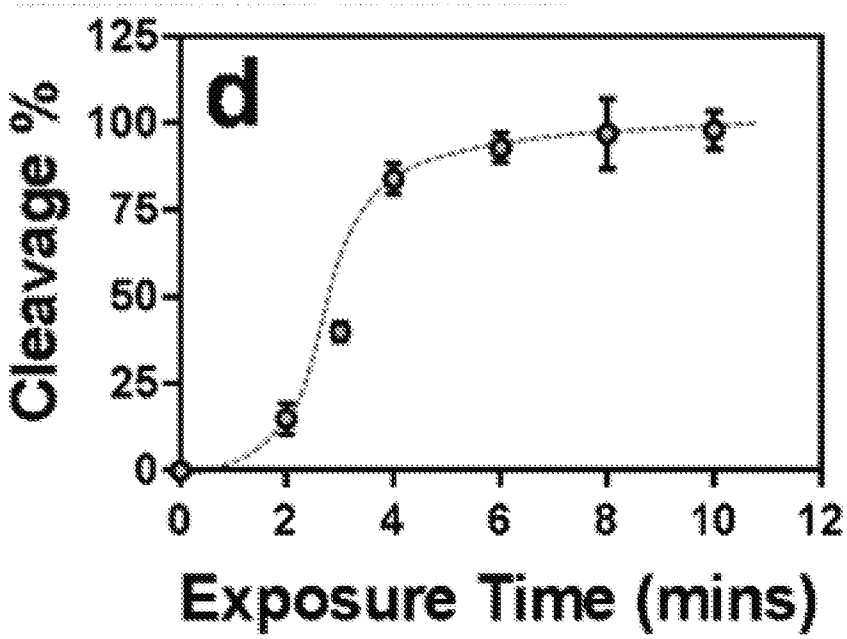

d
Cleavage %
125
100
75
50
25
0
0
2
4
6
8
10
12
Exposure Time (mins)

FIG. 8D

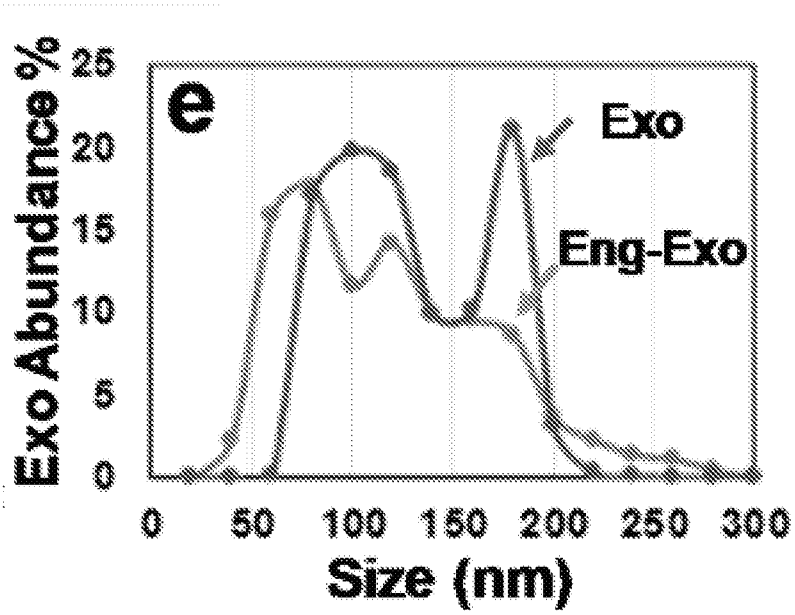

e
Exo
Eng-Exo
Exo Abundance %
25
20
15
10
5
0
0
50
100
150
200
250
300
Size (nm)

FIG. 8E

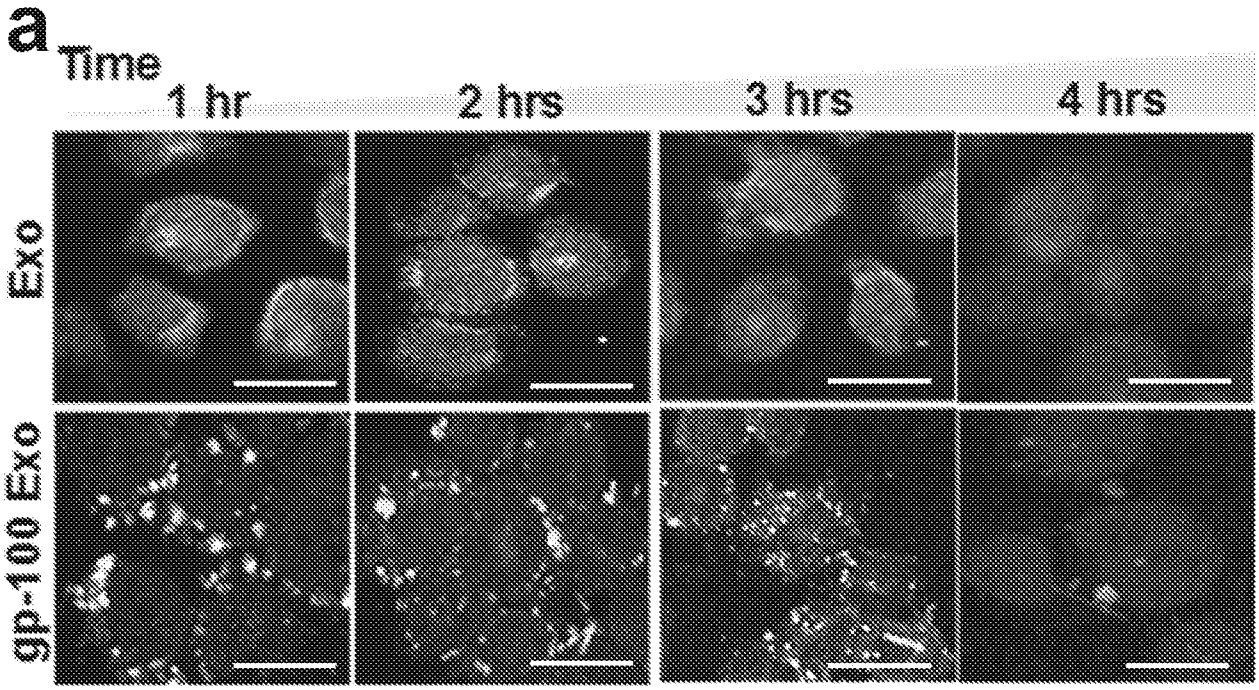

a
Time
1 hr
2 hrs
3 hrs
4 hrs
Exo
gp-100 Exo

FIG. 9A

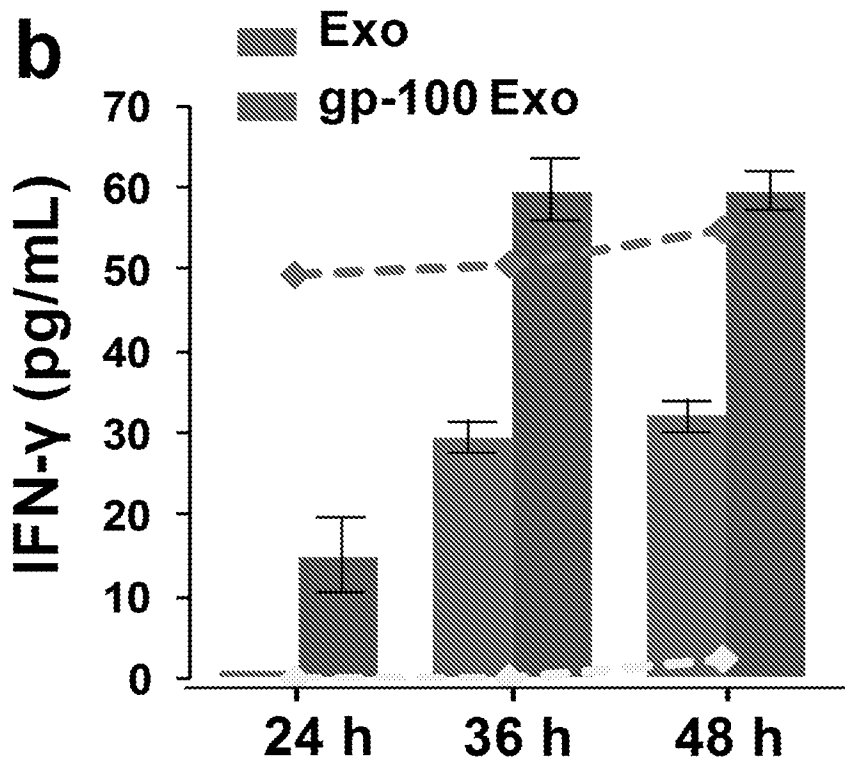


FIG. 9B

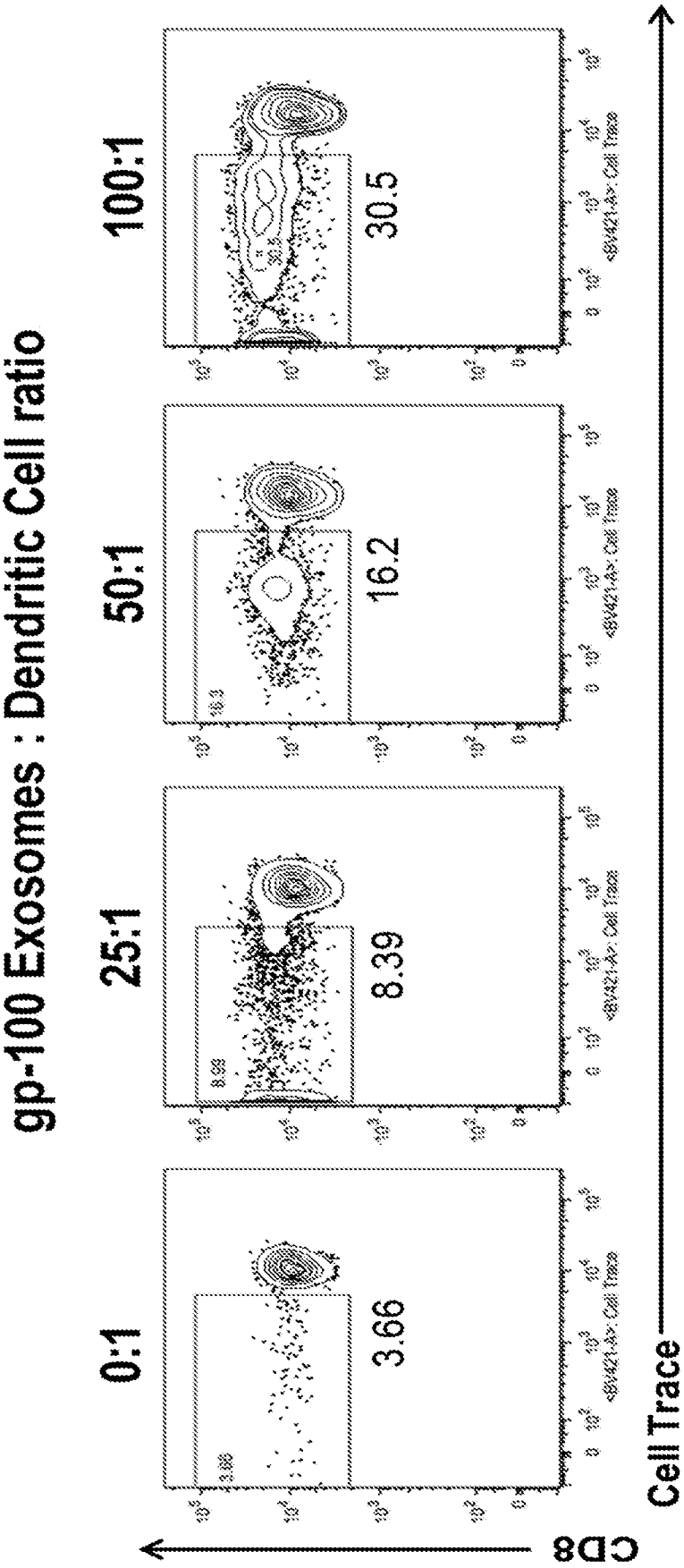

gp-100 Exosomes : Dendritic Cell ratio
0:1
25:1
50:1
100:1
3.66
8.39
16.2
30.5
CD8
Cell Trace

FIG. 10A

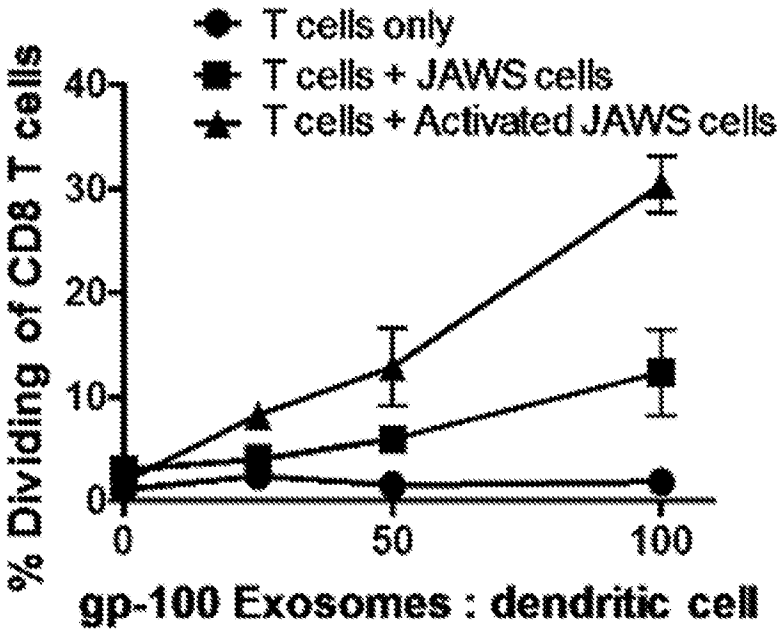

T cells only
T cells + JAWS cells
T cells + Activated JAWS cells
% Dividing of CD8 T cells
40
30
20
10
0
0
50
100
gp-100 Exosomes : dendritic cell

FIG. 10B

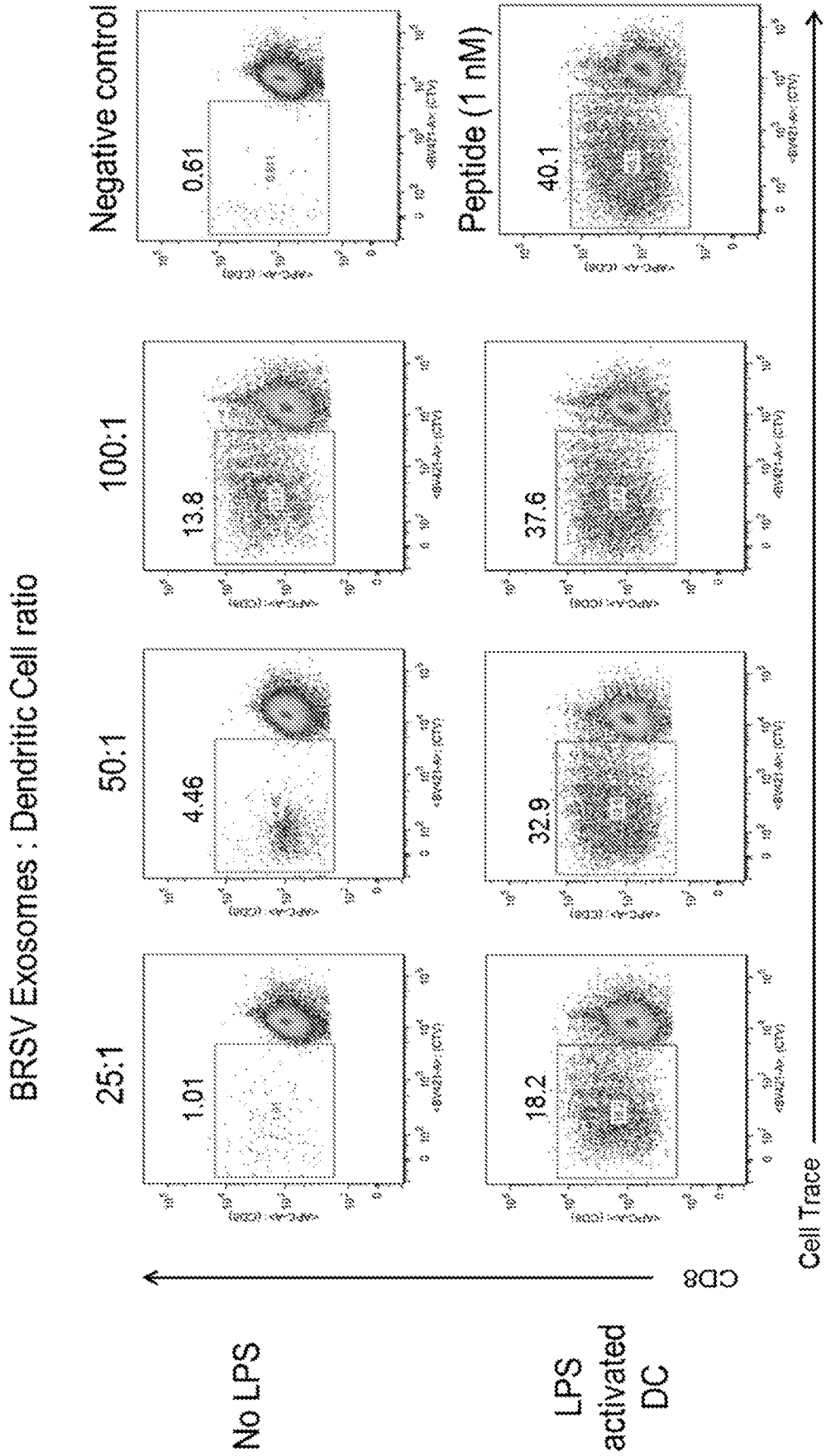

BRSV Exosomes : Dendritic Cell ratio
25:1
50:1
100:1
Negative control
Peptide (1 nM)
No LPS
LPS activated DC
1.01
4.46
13.8
0.61
18.2
32.9
37.6
40.1
CD8
Cell Trace

FIG. 11

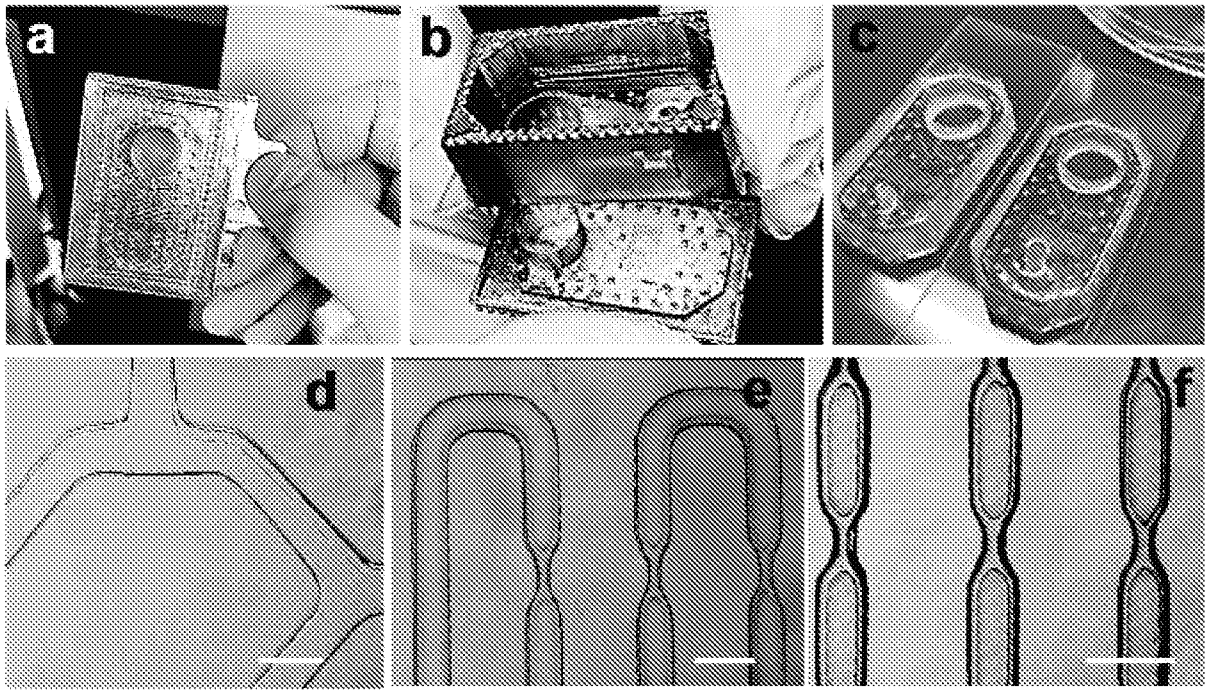


FIG. 12A

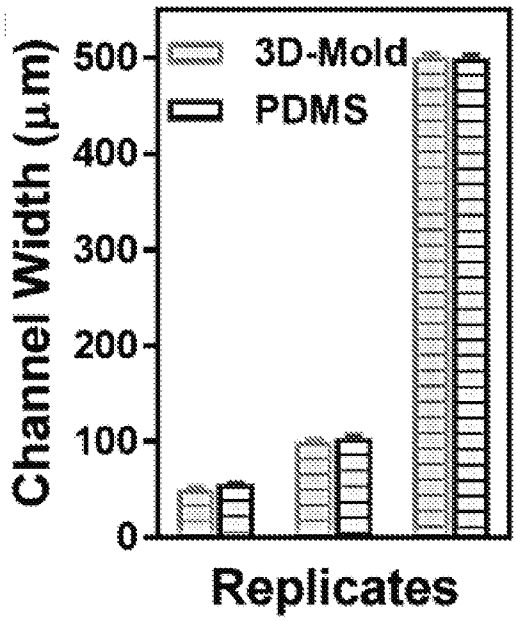


FIG. 12B

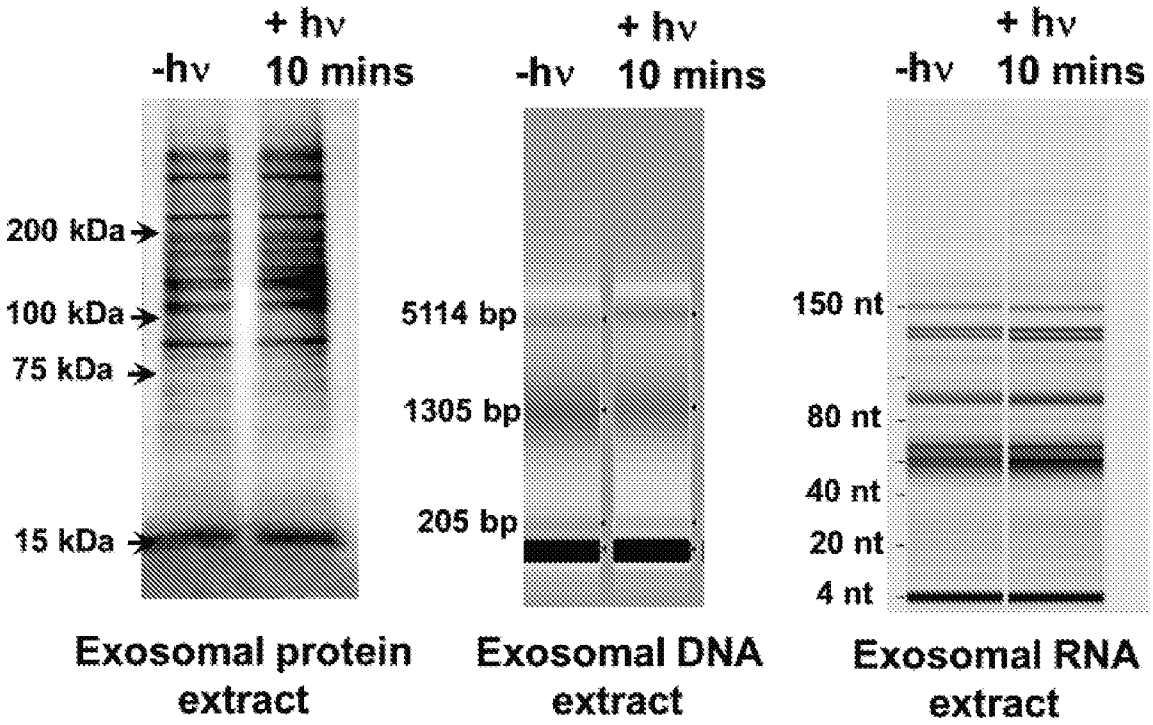

-hν
+ hν 10 mins
200 kDa
100 kDa
75 kDa
15 kDa
Exosomal protein extract
5114 bp
1305 bp
205 bp
Exosomal DNA extract
150 nt
80 nt
40 nt
20 nt
4 nt
Exosomal RNA extract

FIG. 13

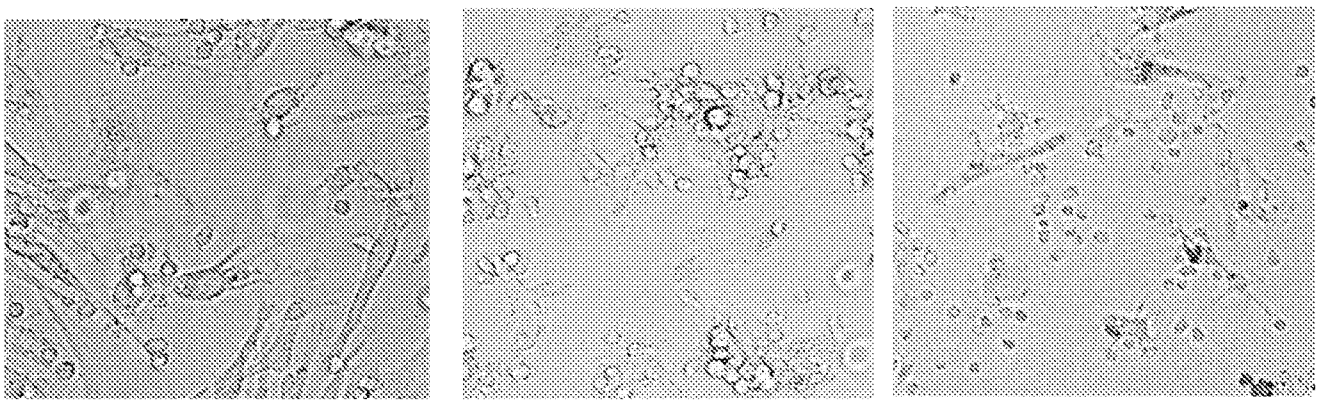

Negative Control
PWM Stimulator
Engineered Exosomes

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/57237

A. CLASSIFICATION OF SUBJECT MATTER

IPC - B03C 1/30, 1/02; G01N 33/48, 33/00 (2019.01)

CPC - B03C 1/015, 1/286, 1/288, 5/028; B01L 3/502761

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZHU et al. 'Microfluidic Engineering of Exosomes: Editing Cellular Messages for Precision Therapeutics' Lab On A Chip Volume, May 7, 2018 Vol. 18, Issue: 12, pp 1-13; page 5, column 1, second paragraph; page 6, column 2, second paragraph; page 7, column 2, second paragraph. DOI: 10.1039/c8lc00246k	1-19, 20/1-19, 21/20/1-19, 22/21/20/1-19, 23/20/1-19, 24/20/1-19, 25/20/1-19, 26/20/1-19, 27-34, 35/27-34, 36/35/27-34, 37/35/27-34, 37/36/35/27-34, 38/38/37/36/35/27-34, 39/37/36/35/27-34, 40/39/37/36/35/27-34, 41-43, 44/43
Y	US 2008/0302732 A1 (SOH et al.) 11 December 2008; figures 1-2; paragraphs [0006], [0011], [0014], [0015], [0041]-[0045], [0060], [0106]	1-19, 20/1-19, 21/20/1-19, 22/21/20/1-19, 23/20/1-19, 24/20/1-19, 25/20/1-19, 26/20/1-19, 27-34, 35/27-34, 36/35/27-34, 37/35/27-34, 37/36/35/27-34, 38/38/37/36/35/27-34, 39/37/36/35/27-34, 40/39/37/36/35/27-34, 41-43, 44/43

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 2019 (23.12.2019)	16 JAN 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/57237

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 45-50
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/57237

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2017/0160294 A1 (Life Technologies As) 08 June 2017; paragraph [0021])	25/20/1-19
Y	US 2012/0183575 A1 (GABRIELSSON) 19 July 2012; abstract; paragraphs [0015], [0074])	36/35/27-34, 37/36/35/27-34, 38/37/36/35/27-34, 39/37/36/35/27-34, 40/39/37/36/35/27-34
Y	US 2010/0165784 A1 (JOVANOVICH et al.) 01 July 2010; figure 6; paragraph [0040]	41-43, 44/43