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(54) Title: METHOD FOR EFFICIENT RADIOLABELING OF BIOMOLECULES

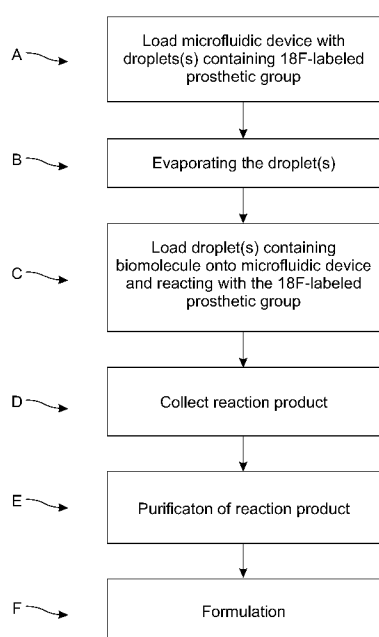


FIG. 1

(57) Abstract: A method of radiolabeling a biomolecule using a microfluidic device includes loading one or more droplets containing a 18F-labeled prosthetic group on or into the microfluidic device and evaporating the droplet(s) containing the 18F-labeled prosthetic group. One or more droplets containing the biomolecule are loaded on or into the microfluidic device and reacts with the 18F-labeled prosthetic group that remains after evaporation. The reaction product is then collected from the microfluidic device. In another embodiment, a droplet containing radiometal ions or a radiometal complex is loaded in the microfluidic device, evaporated, and reacted with a droplet containing a biomolecule derivatized with a chelating group. In yet another embodiment, a droplet containing an 18F-labeled salt is loaded onto the microfluidic device, evaporated, and reacted with a droplet containing a biomolecule conjugated with an isotopic exchange labeling site.



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METHOD FOR EFFICIENT RADIOLABELING OF BIOMOLECULES

Related Application

[0001] This Application claims priority to U.S. Provisional Patent Application No. 62/384,079 filed on September 6, 2016, which is hereby incorporated by reference in its entirety. Priority is claimed pursuant to 35 U.S.C. § 119 and any other applicable statute.

Statement Regarding Federally Sponsored

Research and Development

[0002] This invention was made with Government support under R21 AG049918 and R44 MH097271-02, awarded by the National Institutes of Health. The Government has certain rights in the invention.

Technical Field

[0003] The technical field generally relates to microfluidic methods used for the radiolabeling of biomolecules. In particular, the method has particular application for using small volumes of a peptide or protein for radiolabeling in a microfluidic environment.

Background

[0004] Radiolabeled peptides and antibody fragments provide a means to image disease-specific targets with extremely high specificity via positron emission tomography (PET) or single-photon emission computed tomography (SPECT). Imaging agents have been demonstrated by attaching radioisotope labels to peptides, antibodies, affibodies, aptamers, nanoparticles, viruses, and engineered antibodies. Imaging agents have been labeled with F-18, Ga-68, Cu-64, Zr-89, N-13, O-15, Rb-32, etc. (for positron emission tomography, PET), and Tc-99m, I-123, I-131, In-111, etc. (for single-photon emission computed tomography, SPECT). In addition to imaging agents, radiolabeling of biomolecules can also yield useful therapeutic agents, if labeled with appropriate isotopes such as Y-90 (various cancers and arthritis), Lu-177 (various cancers), I-131 (thyroid cancer), P-32 (abnormal excess of red blood cells), Cs-131, Pd-103, Rd-223, Ra-223 (brachytherapy).

[0005] In a conventional approach to radiolabeling of biomolecules, typically, 100-500 µg of peptide or protein is used in conventional approaches and the reaction is performed in large-scale commercial plastic centrifuge tubes or glass vial. See Z. Wu et al., J Nucl Med, vol. 55:1178 (2014). In more detail, a relatively large amount of the biomolecule sample is

first prepared in a large solution volume (normally 100 - 1000 μ L) in the reaction vessel. Then, the radioisotope-containing intermediate compound is introduced to the reaction vial to label biomolecules further increasing the total volume. This increase in volume reduces the concentration and can adversely affect the rate and/or yield of the labeling reaction in some cases. The vial is then incubated for the desired reaction time (e.g., 10 min to 1 hr), and then the contents are removed for purification, analysis, and injection.

[0006] To reduce the high cost of radiolabeling, efficient techniques with lower biomolecule consumption are necessary. Reduced cost will have benefit in the research and development of disease-specific imaging probes, as well as in the routine clinical use of these agents. Proteins and peptides are very expensive and thus are a significant cost component in radiolabeling reactions. Microfluidic techniques have been used for labeling of biomolecules more recently. For example, Reichert et al. utilized a microfluidic platform made of elastomeric polymer (i.e., polydimethylsiloxane (PDMS) on glass substrate) to radiolabel clinically used antibodies (i.e., Trastuzumab) aiming for faster and more efficient labeling reaction. See B. D. Wright, J. Whittenberg, A. Desai, C. DiFelice, P. J. A. Kenis, S. E. Lapi, and D. E. Reichert, "Microfluidic Preparation of a ^{89}Zr -Labeled Trastuzumab Single-Patient Dose," J. Nucl. Med., vol. 57, no. 5, pp. 747–752, May 2016. Their group has also performed radiolabeling with other isotopes and chelating groups on biomolecules. Even though this method has some advantages of allowing smaller-scale "development" experiments which cannot be performed in macroscale devices, a key shortcoming is that it does not provide a means to concentrate and remove the solvent from the labeling agent, and thus large volumes (similar to macroscale apparatus) are needed to prepare high amounts of the imaging agent or therapeutic (e.g., a single patient dose). These large volumes lead to high consumption of the biomolecule.

[0007] Previously, using a droplet-based microfluidic chip such as disclosed in U.S. Patent No. 9,005,544, it has been shown that reagent consumption for small-molecule PET tracers can be reduced 2-3 orders of magnitude compared to conventional approaches. See also P.Y. Keng and R.M. van Dam, Mol Imaging, vol. 14: 1535 (2015). Due to the fragility of biomolecules, it is desirable that the incorporation of the radioisotope occur under mild conditions (e.g., under physiological temperatures and without organic solvents). A wide variety of strategies for labeling have been developed. For some isotopes, a chelating group is incorporated into the biomolecule under mild conditions beforehand, and then later the isotope is added and chelation is performed under mild conditions. For some isotopes and labeling methods (e.g., Ga-68), higher temperatures are needed to achieve good incorporation into the chelator; such approaches

may not be suitable for all types of biomolecules. For fluorine-18, the typical approach is to synthesize an ^{18}F -labeled “prosthetic group” (the synthesis can be performed under harsh conditions). The prosthetic group contains functional groups that react with a counterpart functional group on the biomolecule under mild conditions. The functional group may already be present in the biomolecule (e.g., amine groups on lysine residues in peptides or proteins for labeling with succinimidyl-ester containing prosthetic group), or it may need to be specially incorporated into the biomolecule beforehand under mild conditions. A third approach is to perform “isotopic exchange” reactions. In this case, the fluorine (in form of stable, non-radioactive F-19) is incorporated into the biomolecule beforehand, and then, under mild conditions, the F-18 isotope is added to the biomolecule whereupon the radioactive and non-radioactive forms are exchanged. While devices such as those described in Keng et al. are used to produce PET tracers they have not been used to radiolabel biomolecules such as proteins or peptides.

Summary

[0008] According to one aspect of the invention, a method of radiolabeling a biomolecule such as a peptide or protein using a microfluidic device or “on chip” is disclosed. The method uses a small volume of peptide or protein which is typically on the order of 100 nL to 20 μL and undergoes reaction at mild conditions. To investigate the feasibility of on chip microscale volume radiolabeling, an example of prosthetic group chemistry was performed using the microfluidic device. A thiol-containing RGD peptide was labeled with fluorine-18 in a site-specific manner via the maleimide-based prosthetic group, N-2-(4-[^{18}F]fluorobenzamido)ethylmaleimide ([^{18}F]FBEM).

[0009] The radiolabeling reaction was performed in droplets located on the microfluidic device. First, a droplet of solution containing the prosthetic group was loaded onto the microfluidic device. Next, the solvent was removed by evaporation at around room temperature. This process is facilitated by the architecture of the microfluidic device. The open structure allows rapid evaporation while the droplet and the contained prosthetic group remain in place on the surface of the microfluidic device. Next, a small droplet of the peptide solution is added, and then the reaction allowed to proceed at room temperature. Optionally, enhancement of reaction perform can likely be achieved by incorporating a mixing process. Examples of techniques for rapid mixing in droplets on surfaces include, for example, electrohydrodynamic methods, acoustic methods, movement of droplets, etc. Reaction might also be enhanced by addition of a small amount of an additive to help solubility of the dried

residue of the prosthetic group. Following reaction, the droplet is removed from the microfluidic device and then undergoes purification and formulation, QC analysis, and then injection.

[0010] In one embodiment, a method of radiolabeling a biomolecule includes providing a microfluidic device; loading a droplet containing a ^{18}F -labeled prosthetic group on or into the microfluidic device; evaporating the droplet containing the ^{18}F -labeled prosthetic group; loading a droplet containing the biomolecule on or into the microfluidic device and reacting with the ^{18}F -labeled prosthetic group; and collecting the reaction product from the microfluidic device.

[0011] In another embodiment, a method of radiolabeling biomolecules includes providing a microfluidic device; loading a plurality of droplets containing a ^{18}F -labeled prosthetic group on or into discrete areas of the microfluidic device; evaporating the plurality of droplets; loading a plurality of droplets each containing the biomolecules on or into the microfluidic device and reacting the plurality of droplets with the ^{18}F -labeled prosthetic group at each discrete area; and collecting the reaction products at each discrete area from the microfluidic device.

[0012] In another embodiment, a method of radiolabeling a biomolecule includes providing a microfluidic device; loading a droplet containing radiometal ions or a radiometal complex on or into the microfluidic device; evaporating the droplet containing the radiometal ions or the radiometal complex; loading a droplet containing the biomolecule derivatized with a chelating group on or into the microfluidic device and reacting with the radiometal ions or the radiometal complex; and collecting the reaction product from the microfluidic device. In some embodiments, the collection of the reaction products may include complete removal from the microfluidic device. In other embodiments, collection may include transferring the products to another downstream processing system on the microfluidic device or coupled thereto.

Brief Description of the Drawings

[0013] FIG. 1 illustrates a sequence of operations for radiolabeling a biomolecule according to one embodiment.

[0014] FIG. 2 illustrates one illustrative example of a basic microfluidic device that can be used to radiolabel a biomolecule.

[0015] FIG. 3 illustrates another illustrative example of an electrowetting-on-dielectric (EWOD) microfluidic device that can be used to radiolabel a biomolecule.

[0016] FIG. 4 illustrates a top down view of a EWOD microfluidic device according to another embodiment.

[0017] FIG. 5 illustrates another embodiment of a microfluidic device that includes multiple reaction sites.

[0018] FIG. 6 illustrates the chemical reaction of a thiol-containing RGD peptide (i.e., cyclo(RGDfC) peptide) labeled with fluorine-18 in a site specific manner via the maleimide-based prosthetic group, N-2-(4-[¹⁸F]fluorobenzamido)ethylmaleimide ([¹⁸F]FBEM).

[0019] FIG. 7 illustrates a top down view of the polytetrafluoroethylene (PTFE)-coated glass chip that was used for the reaction.

[0020] FIG. 8 illustrates the sequence of operations used to radiolabel cyclo(RGDfC) peptide with [¹⁸F]FBEM. Note that in this experiment, the microfluidic device or chip is open to the external environment.

[0021] FIG. 9A illustrates a graph of the of radio HPLC peak of the conjugate, [¹⁸F]FBEM-c(RGDfC).

[0022] FIG. 9B illustrates UV absorbance HPLC peaks for the same reaction using non-radioactive [¹⁸F]FBEM.

[0023] FIG. 10 illustrates a graph of the conjugation efficiency of [¹⁸F]FBEM as a function of peptide mass.

[0024] FIG. 11 illustrates the estimate of the crude radiolabeling yield which was obtained by multiplying the conjugation efficiency by the collection efficiency for the chip. High labeling efficiency (84.1, 83.9, 70.3%) was achieved for 100, 20 and 6.7μg, respectively.

[0025] FIG. 12 illustrates a sequence of operations for radiolabeling a biomolecule according to another embodiment.

[0026] FIG. 13 illustrates a sequence of operations for radiolabeling a biomolecule according to another embodiment.

Detailed Description of the Illustrated Embodiments

[0027] FIG. 1 illustrates one embodiment of a method of radiolabeling a biomolecule. Biomolecule, as used herein, refers to biomolecules that are present in living organisms. Examples of biomolecules that can be radiolabeled include, by way of example, proteins, peptides and fragments thereof, antibodies, antibody fragment, affibodies, aptamers, nanoparticles, dendrimers, viruses, metabolites, and the like. With reference to FIG. 1, as seen in operation A, a microfluidic device 50 is loaded with one or more droplets 12 that contain an 18F-labeled prosthetic group. Prosthetic groups that incorporate 18F are used to

indirectly incorporate ^{18}F into biomolecules such as proteins and peptides via conjugation which can take place under mild conditions. Examples of prosthetic groups include, by way of example, 4-[^{18}F]fluorobenzaldehyde ([^{18}F]FBA), N-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB), 2-bromo-N-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]acetamide ([^{18}F]FPyBrA), 4-nitrophenyl-2-[^{18}F]fluoropropionate ([^{18}F]NPE), 6-[^{18}F]Fluoronicotinic Acid 2,3,5,6-Tetrafluorophenyl Ester ([^{18}F]F-Py-TFP), 4-[^{18}F]fluorobenzyl-2-bromo-acetamide ([^{18}F]FBBA), 4-[^{18}F]fluorobenzylamido-propionyl maleimide ([^{18}F]FBAPM), N-2-(4-[^{18}F]fluorobenzamido)ethylmaleimide ([^{18}F]FBEM), N-[4-[(4-[^{18}F]fluorobenzylidene)aminooxyl]butyl]maleimide ([^{18}F]FBAM), 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME), 1-(4-[^{18}F]fluorophenyl)pyrrole-2,5-dione ([^{18}F]FPPD), N-[3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)phenyl]-4-[^{18}F]fluorobenzamide ([^{18}F]DDPFB), 4-[^{18}F]fluorobenzaldehyde-O-(2-{2-[2-(pyrrol-2,5-dione-1-yl)ethoxy]-ethoxy}-ethyl)oxime ([^{18}F]FBOM), [^{18}F]fluoro phenyloxadiazole methylsulfone ([^{18}F]FPOS), 4-(p-([^{18}F]fluorosulfonyl)phenyl)-1,2,4-triazoline-3,5-dione ([^{18}F]FS-PTAD), 2-cyanobenzothiazole ([^{18}F]CBT), 2-[^{18}F]fluoroethylazide FEAz ([^{18}F]FEA), 1-(3-azidopropyl)-4-(3-[^{18}F]fluoropropyl)piperazine ([^{18}F]AFP), 1-(but-3-ynyl)-4-(3-[^{18}F]fluoropropyl)piperazine ([^{18}F]BFP), 2-deoxy-2-[^{18}F]fluoroglucopyranosyl azide ([^{18}F]FDG- β -Az), 4-[^{18}F]fluoro-N-methyl-N-(prop-2-ynyl)- ([^{18}F]F-SA), O-propargyl-4-[^{18}F]fluorobenzoate ([^{18}F]PFB), 2-[^{18}F]fluoroethyl ester ([^{18}F]FEtO), [^{18}F]azadibenzocyclooctyne ([^{18}F]F-ADIBO), 5-[^{18}F]fluoro-1-pentyne ([^{18}F]FPentyne), 2-[^{18}F]fluoro-3-pent-4-yn-1-yloxy pyridine ([^{18}F]FPyKYNE), [^{18}F]fluoroepibatidine ([^{18}F]FHP), [^{18}F]trans-cyclooctene ([^{18}F]F-TCO). Prosthetic groups bind to a number of different chemical groups that are located on the biomolecule. These include aminooxy, amine, thiol (sulfhydryl), L-tyrosine, L-cysteine groups. In addition, certain prosthetic groups bind to molecules via Click chemistry via alkyne, azide, or tetrazine links.

[0028] For radiochemistry applications, the prosthetic group is typically synthesized using a radiochemistry synthesizer. Typically, many of these radiochemistry synthesizers are automated or have some automated steps can be performed without the aid of human intervention. An example of one such radiochemistry synthesizer is the ELIXYS automated radiochemistry platform that is available from Sofie Biosciences Inc., Culver city, California. It should be understood that the prosthetic group may be formed using any method known to those skilled in the art including both automated operations as discussed above as well as manual synthesis.

[0029] The microfluidic device 50 may include any number of microfluidic devices that are used to handle and manipulate small quantities of reagents. Often, these microfluidic devices 50 resemble or are referred to as chips where various unit operations may be performed thereon. FIG. 2 illustrates one illustrative example of a basic microfluidic device 50. In this embodiment, the microfluidic device 50 includes a substrate 52 which may include a chemically inert material such as glass. In some embodiments where the droplet 12 is aqueous-based, the top surface of the substrate 54 may be coated with thin hydrophobic layer such as polytetrafluoroethylene (PTFE). In some other embodiments, portions of the surface of the substrate 52 may be rendered hydrophilic so that the droplet 12 preferentially flows or sticks to certain surface regions of the substrate 52.

[0030] FIG. 3 illustrates another embodiment of a microfluidic device 50. In this embodiment, the microfluidic device 50 is an electrowetting-on-dielectric (EWOD) based device. The microfluidic device 50 includes a two parallel inert substrates 56, 58 (e.g., glass) with one substrate 56 serving as a bottom plate and the other substrate 58 serving as the top plate. A plurality of individually-addressable actuation electrodes 60 are disposed on the bottom substrate 56 and covered with a dielectric layer 62. The top substrate 58 includes a conductive layer 64 (e.g., indium tin oxide (ITO)) and serves as the ground electrode. The droplets 12 are sandwiched between the two layers 56, 58 as illustrated. A hydrophobic layer 66 coats the exposed surfaces of the two substrates 56, 58 to prevent the droplet 12 from sticking to the surface and enhancing the change in contact angle upon electrical actuation. As seen in FIG. 3, different electrodes 60 are individually-addressable via circuitry 68 that includes a voltage source 69 which is used to manipulate physical location of the droplet 12 on the microfluidic device 50. In this regard, droplets 12 may be moved along the surface of the microfluidic device 50 to various locations. In some embodiments, various processes may take place at these discrete locations on the microfluidic device 50. For example, heating may take place at certain regions on the surface of the microfluidic device 50 to aid in evaporating the solvent containing the ¹⁸F-labeled prosthetic group as seen, for example in FIG. 4.

[0031] FIG. 4 illustrates another embodiment of a EWOD microfluidic device 50 that includes a plurality of individually-addressable electrodes 60. In this embodiment, the microfluidic device 50 includes a heater 70 integrated into the device itself (e.g., substrate 56). The heater 70 may include series of multiple concentrically arranged resistive heaters that generate heat in response to an applied current. The heater 70 in this embodiment, may be used to accelerate the evaporation of the droplet 12 during the radiolabeling process. In

the embodiment of FIG. 4, one edge of the microfluidic device 50 acts as a loading site 72 while the opposing edge acts as an extraction site 74. For example, droplets 12 can be loaded onto the microfluidic device 50 at the loading site 72 and the droplets 12 are moved under actuation of the electrodes 60 to the heater 70. The heater 70 may be used to aid in evaporation or accelerating reactions under elevated temperatures. Once complete, the droplet that contains the crude reaction product(s) can then be moved again using actuation of the electrodes 60 to move the droplet 12 to the extraction site 74 where the droplet 12 can be removed or transferred from the microfluidic device 50 to another locations or operation for additional processing such as purification and formulation.

[0032] Referring back to FIG. 1, after the microfluidic device 50 is loaded with one or more droplets 12 that contain an 18F-labeled prosthetic group, the droplet 12 is evaporated as seen in operation B. Note that the loading of the droplet 12 may occur manually or automatically and may utilize any known droplet dispensing process or mechanism known in the art. For example, a pipette or other dispenser may be used to load the droplets 12. The droplets 12 may also be directly loaded onto the microfluidic device 50 using side loading like that illustrated in FIG. 4 or even an on-chip loading hole or aperture that receives fluid to form the droplet 12 thereon. The evaporation may take place under the application of heat. For example, a heater 70 such as that disclosed in FIG. 4 may be used to aid in evaporating the solvent. Alternatively, heat may be applied by placing the microfluidic device 50 in contact with a heater or hot surface. The ambient temperature surrounding the droplet 12 may also be heated to accelerate the evaporation process. In one embodiment, the droplet 12 is completely dried so as to leave the dried 18F-labeled prosthetic group on the microfluidic device 50. Alternatively, on other embodiments, the droplet 12 may be partially dried. Evaporation thus encompasses both complete and partial evaporation of the drop 12.

[0033] Next, as seen in operation C, a separate droplet 12 (or multiple droplets 12) that contain the biomolecule(s) to be radiolabeled is loaded on the microfluidic device and reacts with the 18F-labeled prosthetic group that remains on the microfluidic device 50 after evaporation. In some embodiments, the droplet 12 is loaded directly onto the site of the microfluidic device 50 that contains the 18F-labeled prosthetic group. In other embodiments, such as the EWOD-based devices of FIGS. 3 and 4, the droplet 12 may be loaded on or into the microfluidic device 50 as explained above and then moved to the site that contains the 18F-labeled prosthetic group. The droplet 12 may be moved, for example, using the electrodes 60. The droplet 12 that contains the biomolecule(s) may optionally incorporate therein a minimal amount (e.g., a few microliters) of solvent in some instances (e.g.,

acetonitrile or MeCN). In other embodiments, a separate droplet 12 that contains a solvent may be loaded in or onto the microfluidic device 10 and moved to the site that contains the ^{18}F -labeled prosthetic group followed by merging with a separate droplet 12 that contains the biomolecule(s). The volume of the droplet 12 that contains the biomolecule(s) may vary but is typically between about 100 nL and 20 μL . The amount of biomolecule(s) may also be small. For example, as illustrated herein in experimental results good conjugation efficiency with as little as 10 μg of peptides. In one embodiment, the droplet 12 contains less than 100 μg of the biomolecule. In another embodiment, the droplet 12 contains between 10 μg and 50 μg of the biomolecule.

[0034] The reaction between the ^{18}F -labeled prosthetic group and the biomolecule(s) may occur at room temperature in some embodiments. In some embodiments, the biomolecule(s) may be derivatized for site-specific linking of the prosthetic group. Alternatively, linking may be random. In other embodiments, the reaction may be accelerated by the application of heat using, for example, a heater 70 or other heating method disclosed herein. The actual reaction time may vary but is typically on the order of minutes (e.g., 30 minutes). After the elapse of sufficient time for reaction, the crude radiolabeled biomolecules are then collected as illustrated in operation D. Collection of the crude radiolabeled biomolecules may be accomplished by direct retrieval of the droplet 12 from the microfluidic device 50.

Alternatively, the droplet 12 that contains the crude radiolabeled biomolecules may be moved off-chip using the electrodes 60 to the extraction site 74. The droplet 12 containing the crude radiolabeled biomolecules may then be subject to purification using conventional high performance liquid chromatography (HPLC), solid-phase extraction using Sep-Pak, spin column separation, or other purification methods known to those skilled in the art as seen in operation E. The desired fraction(s) obtained in the HPLC purification process can then be, in some embodiments, formulated for use in a subject as seen in operation F of FIG. 1. For example, the sample may be diluted with water or saline to reduce the concentration of any residual organic solvents to acceptable levels for use in humans or other mammals. In addition, techniques such as solid-phase extraction may be performed to exchange one solvent or buffer for another.

[0035] FIG. 5 illustrates a microfluidic chip 50 that is used in connection with another method of radiolabeling biomolecules. In this method, the microfluidic chip 50 contains a number of different sites 80-1, 80-2, 80-3, 80-4, 80-5, 80-6, 80-7, 80-8, 80-9 for accommodating droplets 12. Of course, this embodiment is illustrative and fewer or more sites may be used. For example, these different sites 80-1, 80-2, 80-3, 80-4, 80-5, 80-6, 80-7,

80-8, 80-9 may be used for evaporation of solvent containing the prosthetic group (or other reactants such as radiometal ions, radiometal complexes, ^{18}F -labeled salt as described herein) as well as reaction with the droplet(s) 12 containing the biomolecule(s). In this embodiment, a plurality of droplets 12 containing a ^{18}F -labeled prosthetic group are loaded on or into discrete areas 80-1, 80-2, 80-3, 80-4, 80-5, 80-6, 80-7, 80-8, 80-9 of the microfluidic device 50. In some instances, each droplet 12 at each site 80-1, 80-2, 80-3, 80-4, 80-5, 80-6, 80-7, 80-8, 80-9 contains the same ^{18}F -labeled prosthetic group. For example, if large volumes of final product are desired, parallel reactions involving multiple droplets 12 can be performed and combined or merged after reaction. Alternatively, each droplet 12 at each site 80-1, 80-2, 80-3, 80-4, 80-5, 80-6, 80-7, 80-8, 80-9 contains a different ^{18}F -labeled prosthetic group. This embodiment is thus able to create different radiolabeled biomolecules using a single chip. In yet another alternative, droplets 12 containing different biomolecules may be loaded onto the different sites 80-1, 80-2, 80-3, 80-4, 80-5, 80-6, 80-7, 80-8, 80-9 that contain the same or even different ^{18}F -labeled prosthetic groups. The reacted droplets 12 containing the radiolabeled biomolecules would be collected as previously described. In this embodiment, the microfluidic chip 50 may be created with a large number of sites (e.g., 80-1, 80-2, 80-3, 80-4, 80-5, 80-6, 80-7, 80-8, 80-9) loaded with different labelled prospective or candidate biomolecules which can then be further analyzed with *in vitro* or *in vivo* measures to determine those that exhibit the most desirous properties or efficacy. In this regard, the microfluidic chip 50 may be used as an investigational screening tool for new radiolabeled biomolecules.

[0036] In addition using ^{18}F -labeled prosthetic group, the microfluidic device 50 may also be used in connection with radiometal chelation. This process is illustrated in FIG. 12. In this embodiment, one or more droplets 12 containing radiometal ions or radiometal complexes (like ^{18}F AlF) are loaded onto the microfluidic device 50 and then subject to evaporation as explained herein. Next, one or more droplets 12 containing a biomolecule that is derivatized with the chelating group would then be added and reacted with the dried radiometal ions or complex. The crude reaction produce would then need to undergo purification using HPLC or solid-phase extraction using Sep-Pak or the like and formulation. Examples of chelator groups that may be used with radioactive metal isotopes include, but are not limited to, Desferrioxamine B, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid, 4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane, 1,4,7,10-tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane, 2-[(carboxymethyl)]-[5-(4-nitrophenyl)-1-[4,7,10-tris-(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]pentan-2-yl]-

amino]acetic acid, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid, 4,11-bis-(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]-hexadecane, 1-N-(4-Aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]-eicosane-1,8-diamine, 1,4,7-triazacyclononane-1,4,7-triacetic acid, {4-[2-(bis-carboxymethylamino)-ethyl]-7-carboxymethyl-[1,4,7]triazonan-1-yl}-acetic acid, N,N',N'', tris(2-mercaptoethyl)-1,4,7-triazacyclononane diethylenetriaminepentaacetic acid, 2-(p-isothiocy anatobenzyl)-cyclohexyldiethylenetriaminepentaacetic acid, 1,4,7-triazacyclononane-1,4,7-tris[methyl(2-carboxyethyl)phosphinic acid], 1,4-bis (hydroxycarbonyl methyl)-6-[bis(hydroxycarbonyl methyl)] amino-6-methyl perhydro-1,4-diazepine, 1,2-[[6-(carboxy)-pyridin-2-yl]-methylamino]ethane, N,N'-bis(6-carboxy-2-pyridylmethyl)-ethylenediamine-N,N'-diacetic acid, N,N'-[1-benzyl-1,2,3-triazole-4-yl]methyl-N,N'-[6-(carboxy)pyridin-2-yl]-1,2-diaminoethane, N,N'-[[6-(carboxy)pyridin-2-yl]methyl]-diethylenetriamine-N,N',N''-triacetic acid, N,N'-bis(2-hydroxybenzyl)-ethylenediamine-N,N'-diacetic acid, N,N'-bis(2-hydroxy-5-sulfobenzyl)-ethylenediamine-N,N'-diacetic acid, 4-acetylamino-4-[2-[(3-hydroxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-yl)methyl]-carbamoyl]-ethyl]-heptanedioic acid bis-[(3-hydroxy-1,6-dimethyl-4-oxo-1,4-dihydro-pyridin-2-yl)methyl]-amide], 3,6,9,15-tetraazabicyclo[9.3.1]-pentadeca-1(15),11,13-triene-3,6,9,-triacetic acid, N,N'-(methylenephosphonate)-N,N'-[6-(methoxycarbonyl)pyridin-2-yl]-methyl-1,2-diaminoethane, 1,4,7,10,13,16-hexaazacyclohexadecane-N,N',N'',N''',N''',N''''-hexaacetic acid, 1,4,7,10,13-pentaazacyclopentadecane-N,N',N'',N''',N''''-pentaacetic acid, and tris(2-mercaptobenzyl)amine, 9-hydroxy-2,4-dipyridin-2-yl-3,7-bis(pyridin-2-ylmethyl)-3,7-diazabicyclo-[3.3.1]nonane-1,5-dicarboxylic acid. Exemplary radioactive isotopes include radiometal ions or radiometal complexes such as Cu-64, Ga-67/68, Sc-44/47, In-111, Lu-177, Y-86/90, Bi-213, Pb-212, Ac-225, Zr-89, or [F-18]AlF complex.

[0037] In yet another embodiment, the microfluidic device 50 may also be used in connection with isotopic exchange reactions. This process is illustrated in FIG. 13. In this embodiment, one or more droplets 12 containing ¹⁸F-labeled salt (e.g., [¹⁸F]NaF or other salts as well as phase transfer catalysts such as Kryptofix® 2.2.2 (K-222), TBAHCO₃, and the like) is first loaded onto the microfluidic device 50 followed by evaporation as described herein. A derivatized biomolecule contained in one or more droplets 12 are then added to the microfluidic device 50 and reacted with the dried ¹⁸F-labeled salt. The crude reaction produce would then need to undergo purification using HPLC or solid-phase extraction using Sep-Pak or the like and formulation.

[0038] The biomolecule is conjugated in advance with the isotopic exchange labeling site which may include trifluoroborates (R-BF₃). Various trifluoroborates exist including ArB₃ (aryl construct), AmBF₃ (amino construct), and the like. See, e.g., D. Perrin, *Accounts of chemical research* 49: 1333-1343, 2016, which is incorporated by reference herein. Another isotopic exchange process utilized silicon fluoride acceptor chemistry (SiFA). In this approach there is a single Si-F where the isotopic exchange reaction can happen. Functional groups attached to the Si atom affect reactivity, stability, etc. Examples of common function groups that are attached to Si include p-(di-tert-butylfluorosilyl)--, N-(4-(di-tert-butylfluorosilyl)benzyl)--, etc. Additional examples may be found in Bernard-Gauthier et al. *BioMed Res. Intl.* vol. 2014: 454503, 2014, which is incorporated by reference herein.

[0039] Experimental

[0040] Experiments were conducted on a microfluidic device like that illustrated in FIG. 2. The microfluidic device that was used was a polytetrafluoroethylene (PTFE) coated glass chip that was formed using a glass slide that was treated with fluorosilane as an adhesion promoter overnight in a vacuum desiccator, and then spin-coated (@ 1000 rpm) with a 1% solution of Teflon® AF 2400, followed by baking and annealing (10 min 160°C and 10min at 245°C on a hot plate, then 340°C for 3.5hr in a carbolite oven) to create a 100 nm thick hydrophobic surface. [¹⁸F]FBEM was synthesized on an automated radiochemistry synthesizer (ELIXYS, Sofie Biosciences Inc., Culver city, CA) with a high radiochemical yield (35±8%, decay-corrected) in 100min using a previously reported method as disclosed in W. Cai et al., *J. Nucl. Med.*, vol. 47:1172–1180 (2006), which is incorporated by reference. Purified [¹⁸F]FBEM was formulated in a volatile solvent (dichloromethane) to ensure rapid removal of solvent was possible on the chip. FIG. 6 illustrates the chemical reaction of a thiol-containing RGD peptide (i.e., cyclo(RGDfC) peptide) labeled with fluorine-18 in a site specific manner via the maleimide-based prosthetic group, N-2-(4-[¹⁸F]fluorobenzamido)ethylmaleimide ([¹⁸F]FBEM).

[0041] The on-chip radiolabeling of cyclo(RGDfC) peptide with [¹⁸F]FBEM was performed as illustrated in the operations of FIG. 8. Using a manual pipette, a droplet of [¹⁸F]FBEM that was approximately 20 µL of [¹⁸F]FBEM in dichloromethane (DCM) was loaded onto the chip 50 and concentrated (by evaporation of solvent at room temperature) on a small part of the reaction site of the Teflon-coated chip 50. After the addition of a small (2 µL) of MeCN to dissolve the dried [¹⁸F]FBEM, about 20 µL peptide solution was added for the room temperature conjugation reaction (~ 30 minutes). The effect of peptide concentration was explored by using peptide solution droplets containing different amounts of peptide (ranging from 0.2 to 100 µg). The crude reaction product was then collected from

the chip for analysis via high performance liquid chromatography (HPLC). FIG. 7 illustrates a top down view of the polytetrafluoroethylene (PTFE)-coated glass microfluidic device or chip 50 that was used for the reaction. A droplet 12 is illustrated in the reaction site 13 (4 mm in diameter). Note that in this experiment, the microfluidic device or chip 50 is open to the external environment. In other alternative embodiments, as explained herein, the microfluidic chip may have, for example, two layers with a space located between where the droplet is located (e.g., FIG. 4). The droplet may be moved or manipulated by known fluidic methods or electrowetting-on-dielectric (EWOD) processes (e.g., using an EWOD chip). Evaporation may be conducted in ambient conditions (e.g., air dry) or using the application of an external heat source (e.g., electrode, hot plate, or the like).

[0042] The radiochemical conversion efficiency was estimated based on the area under curve (AUC) of radio HPLC peak of the conjugate, [^{18}F]FBEM-c(RGDfC) as seen in FIG. 9A. FIG. 9B illustrates UV absorbance HPLC peaks for the same reaction using non-radioactive [^{18}F]FBEM. The crude radiolabeling yield (an estimate of isolated yield) was calculated by multiplying radiochemical conversion (%) (i.e., conjugation efficiency) by the sample collection efficiency from the chip. FIG. 10 illustrates conjugation efficiency as a function of peptide mass. With reference to FIG. 11, which illustrates the crude radiolabeling yield, on-chip [^{18}F]FBEM conjugation of c(RGDfC) resulted in high labeling efficiency (84.1, 83.9, 70.3% for 100, 20 and 6.7 μg , respectively). Even using as little as 2 μg peptide exhibited practical conjugation efficiency (26.6%). The sample collection efficiency from the chip was over 90%, the residual being stuck on the chip. Cerenkov imaging and radioactivity measurements confirmed there were negligible losses during other steps of the on-chip labeling.

[0043] Microscale radiolabeling of peptides using low amount of peptide, protein, or biomolecules is feasible using droplet microfluidics. Peptide consumption is reduced 50-100x compared to conventional methods. Although the current study used $\sim 1\text{mCi}$ of [^{18}F]FBEM per experiment, the activity could be scaled up by repeated [^{18}F]FBEM loading or by pre-concentrating [^{18}F]FBEM using a method like microfluidic membrane distillation. The small volume approach described herein may also be applied to the development of antibody-based imaging probes and radiotherapeutics with reduced precursor consumption and increase of effective specific activity.

[0044] In addition to c(RGDfC), labeling was performed of an engineered antibody compound, Cys-Diabody (55kDa) which is smaller than the size of regular antibodies for accelerated clearance from blood circulation but maintaining a tumor-specificity. The Cys-Diabodies, which contain sulfhydryl (thiol) residues after reduction of the disulphide bridge,

were labeled with [^{18}F]FBEM. Because labeling occurs only at the Cys residues, labeling occurs in a site-specific manner, i.e. at a well-controlled/predicted reaction site in a molecule. This is important so that the labeling does not interfere with the biological activity of the molecules (generally assessed via an immunoreactivity assay for antibodies and antibody fragments).

[0045] The methods described herein can be scaled-up for larger capacity. Multiple reaction sites can be easily be incorporated to the microfluidic device or chip for multiple reactions in parallel as explained herein. This would enable synthesizing a higher dose of imaging probes by combining or merging the product from the multiple reaction sites together. In addition, this method can be used for high-throughput screening. This parallel reaction approach could also be used to generate a number of different imaging agents in parallel, i.e., by labeling a different biomolecule at each of several different sites. To purify all the different molecules, one could combine the platform with a high-through purification/analysis system such as UHPLC. Furthermore, high-throughput imaging systems could enable rapid evaluation of all the candidate molecules in a short period of time. Various reaction conditions (e.g., reagent ratio, temperature, reaction time) can be tested in one chip simultaneously. In that way, one can save the tremendous amount of time required for the development of new imaging probes and radiotherapeutics. Normally such optimization experiments are performed at a rate of one, or a small number, per day.

[0046] While reagents were loaded manually by pipette for initial experiments, addition of reagents could be performed automatically using a variety of pumping, pipetting, or droplet manipulation technologies. In addition, while experiments were performed at room temperature, evaporation and/or reaction steps may be performed at elevated temperature by placing the chip in thermal contact with a temperature control device or incorporating heaters into the device. This may enable more rapid processing that would improve the yield (by minimizing radioactive decay). The prosthetic group can be pre-concentrated to allow more radioactivity to “fit” within the volume capacity of the chip, thus producing a higher dose of the labeled biomolecule. This would enable the production of higher “doses” that may be suitable for imaging in humans (or even to make batches suitable for imaging many patients). Though purification is currently performed off-chip, there are several chip-based chemical purification methods that have been developed (e.g., solid-phase extraction, flowing through a purification resin-filled channel, etc.) that may be suitable for on-chip purification. Similarly, analysis of the labeled product (to assess pH, chemical purity, radiochemical purity, etc.) is performed off-chip with conventional instrumentation, but chip-based

techniques are being developed that could enable this to be performed on-chip as well, ultimately enabling a fully-integrated chip for production, purification, and QC testing of radiolabeled biomolecules.

[0047] While embodiments of the present invention have been shown and described, various modifications may be made without departing from the scope of the present invention. The invention, therefore, should not be limited except to the following claims and their equivalents.

What is claimed is:

1. A method of radiolabeling a biomolecule comprising:
providing a microfluidic device;
loading a droplet containing a ^{18}F -labeled prosthetic group on or into the microfluidic device;
evaporating the droplet containing the ^{18}F -labeled prosthetic group;
loading a droplet containing the biomolecule on or into the microfluidic device and reacting with the ^{18}F -labeled prosthetic group; and
collecting the reaction product from the microfluidic device.
2. The method of claim 1, wherein the reaction product is purified and formulated for administration to a mammal.
3. The method of claim 1, wherein the biomolecule comprises a protein or peptide.
4. The method of claim 3, wherein the biomolecule comprises an antibody, antibody fragment, affibody, aptamer, nanoparticle, dendrimer, or virus.
5. The method of claim 1, wherein the droplet contains less than 100 μg of the biomolecule.
6. The method of claim 1, wherein the droplet contains between 10 μg and 50 μg of the biomolecule.
7. The method of claim 1, wherein the steps of loading the droplet containing a ^{18}F -labeled prosthetic group on or into the microfluidic device and evaporating the droplet are repeated a plurality of times.
8. The method of claim 1, wherein evaporating the droplet comprises heating the droplet.

9. The method of claim 1, wherein the ^{18}F -labeled prosthetic group comprises N-2-(4-[^{18}F]fluorobenzamido)ethylmaleimide ([^{18}F]FBEM).
10. The method of claim 1, wherein the microfluidic device comprises an electrowetting-on-dielectric (EWOD) device.
11. The method of claim 1, wherein the microfluidic device comprises a polytetrafluoroethylene (PTFE) coated glass.
12. A method of radiolabeling biomolecules comprising:
providing a microfluidic device;
loading a plurality of droplets containing a ^{18}F -labeled prosthetic group on or into discrete areas of the microfluidic device;
evaporating the plurality of droplets;
loading a plurality of droplets each containing the biomolecules on or into the microfluidic device and reacting the plurality of droplets with the ^{18}F -labeled prosthetic group at each discrete area; and
collecting the reaction products at each discrete area from the microfluidic device.
13. The method of claim 12, wherein the plurality of droplets containing the ^{18}F -labeled prosthetic group contain the same prosthetic group.
14. The method of claim 12, wherein the plurality of droplets containing the ^{18}F -labeled prosthetic group contain a different prosthetic group.
15. The method of claim 12, wherein the plurality of droplets containing the biomolecules contain the same biomolecule.
16. The method of claim 12, wherein the plurality of droplets containing the biomolecules contain a different biomolecule.
17. The method of claim 12, wherein the reaction products are purified and formulated for administration to a mammal.

18. A method of radiolabeling a biomolecule comprising:
providing a microfluidic device;
loading a droplet containing radiometal ions or a radiometal complex on or into the microfluidic device;
evaporating the droplet containing the radiometal ions or the radiometal complex;
loading a droplet containing the biomolecule derivatized with a chelating group on or into the microfluidic device and reacting with the radiometal ions or the radiometal complex; and
collecting the reaction product from the microfluidic device.
19. The method of claim 18, wherein the biomolecule comprises a protein or peptide.
20. The method of claim 18, wherein the biomolecule comprises an antibody, antibody fragment, affibody, aptamer, nanoparticle, dendrimer, or virus.
21. The method of claim 18, wherein the droplet contains less than 100 μg of the biomolecule.
22. The method of claim 18, wherein the droplet contains between 10 and 50 μg of the biomolecule.
23. The method of claim 18, wherein a plurality of droplets containing radiometal ions or a radiometal complex are loaded on different sites of the microfluidic device and wherein a plurality of droplets containing the biomolecule derivatized with a chelating group are loaded on or into the microfluidic device and are reacted with the radiometal ions or the radiometal complex at the different sites.
24. A method of radiolabeling a biomolecule comprising:
providing a microfluidic device;
loading a droplet containing an ^{18}F -labeled salt on or into the microfluidic device;
evaporating the droplet containing the ^{18}F -labeled salt;

loading a droplet containing the biomolecule conjugated with an isotopic exchange labeling site on or into the microfluidic device and reacting with the ^{18}F -labeled salt; and collecting the reaction product from the microfluidic device.

25. The method of claim 24, wherein the biomolecule comprises a protein or peptide.

26. The method of claim 24, wherein the biomolecule comprises an antibody, antibody fragment, affibody, aptamer, nanoparticle, dendrimer, or virus.

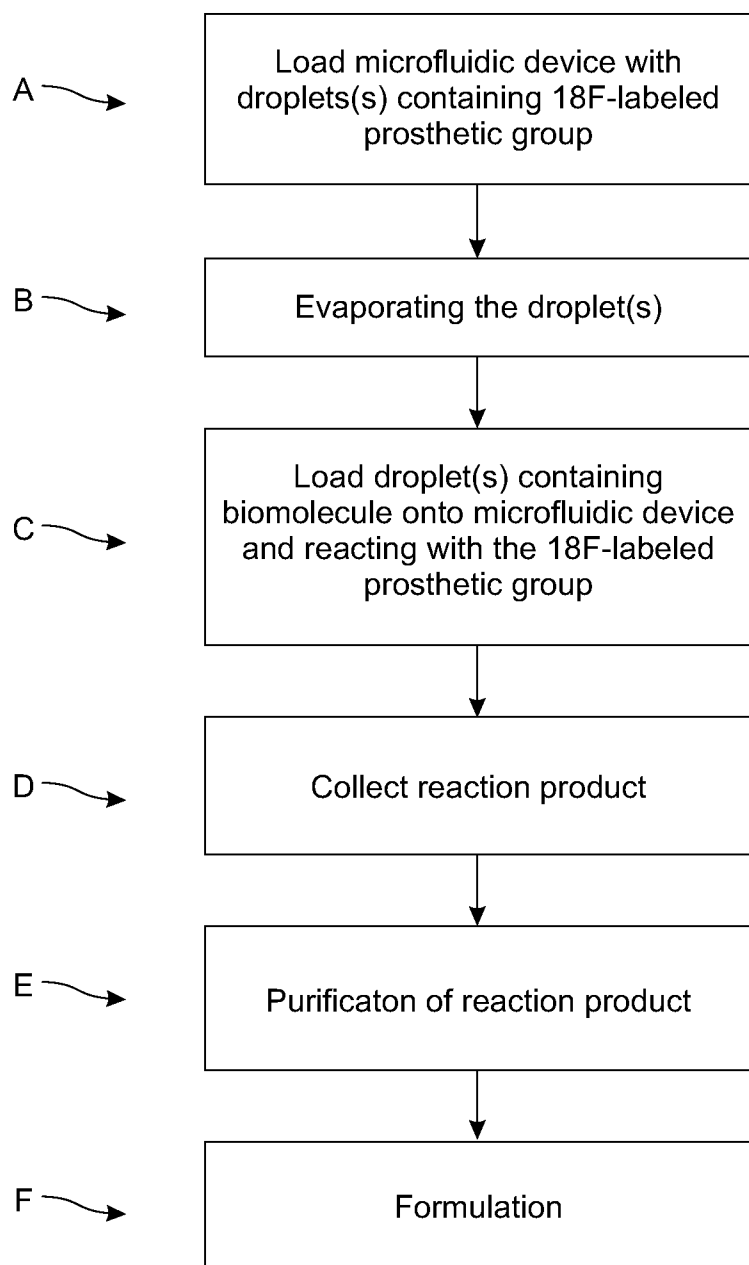
27. The method of claim 24, wherein the droplet contains less than 100 μg of the biomolecule.

28. The method of claim 24, wherein the droplet contains between 10 and 50 μg of the biomolecule.

29. The method of claim 24, wherein a plurality of droplets containing the ^{18}F -labeled salt are loaded on different sites of the microfluidic device and wherein a plurality of droplets containing the biomolecule conjugated with the isotopic exchange labeling site are loaded on or into the microfluidic device and are reacted with the ^{18}F -labeled salt at the different sites.

30. The method of any of claims 18 or 24, further comprising purifying the reaction product and formulating the reaction product for administration to a mammal.

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*FIG. 1*

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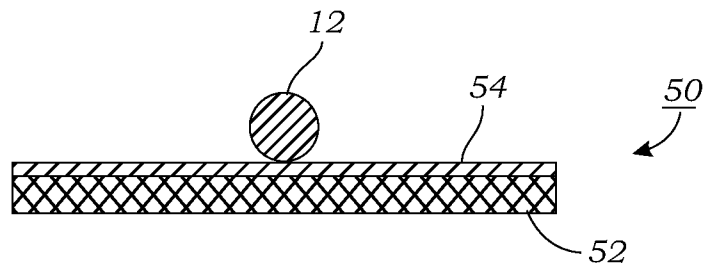


FIG. 2

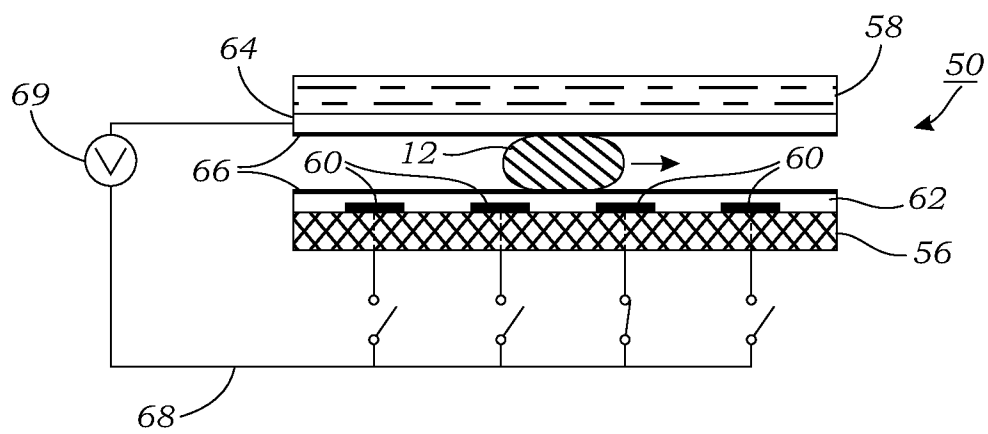


FIG. 3

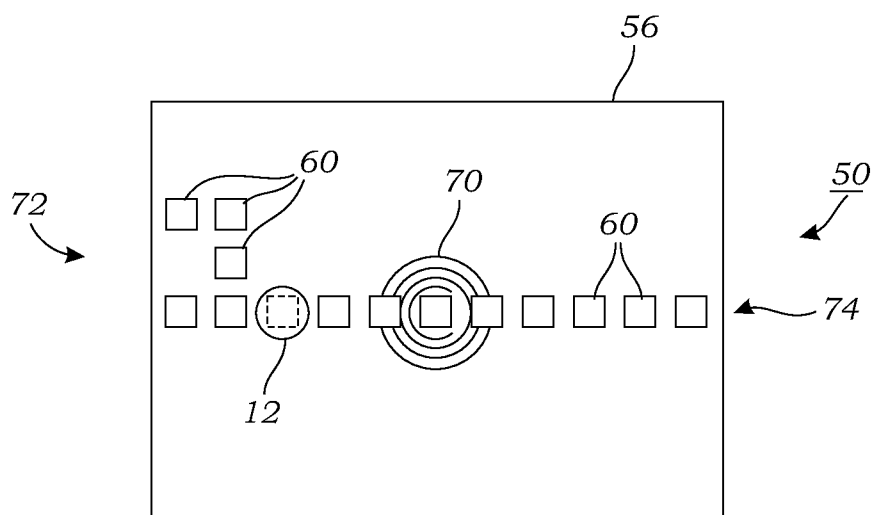


FIG. 4

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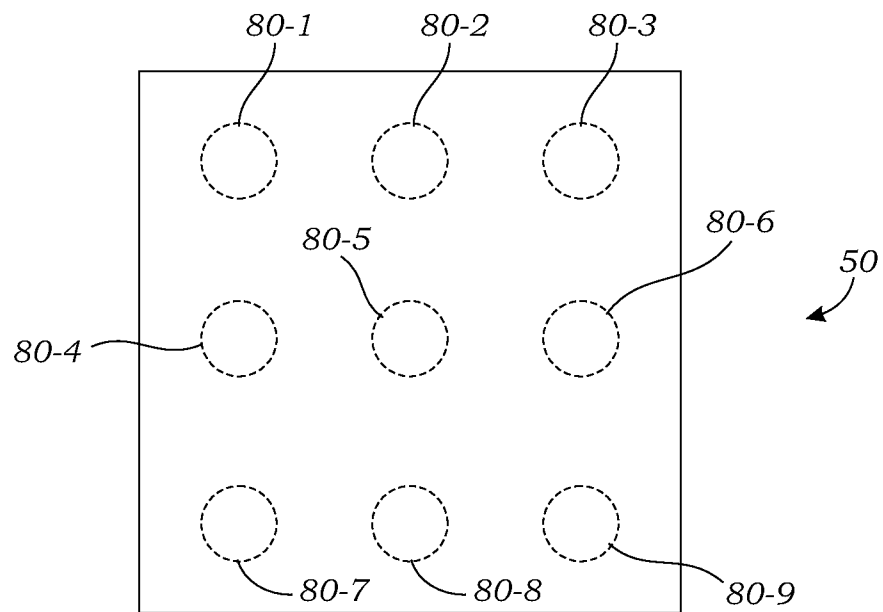


FIG. 5

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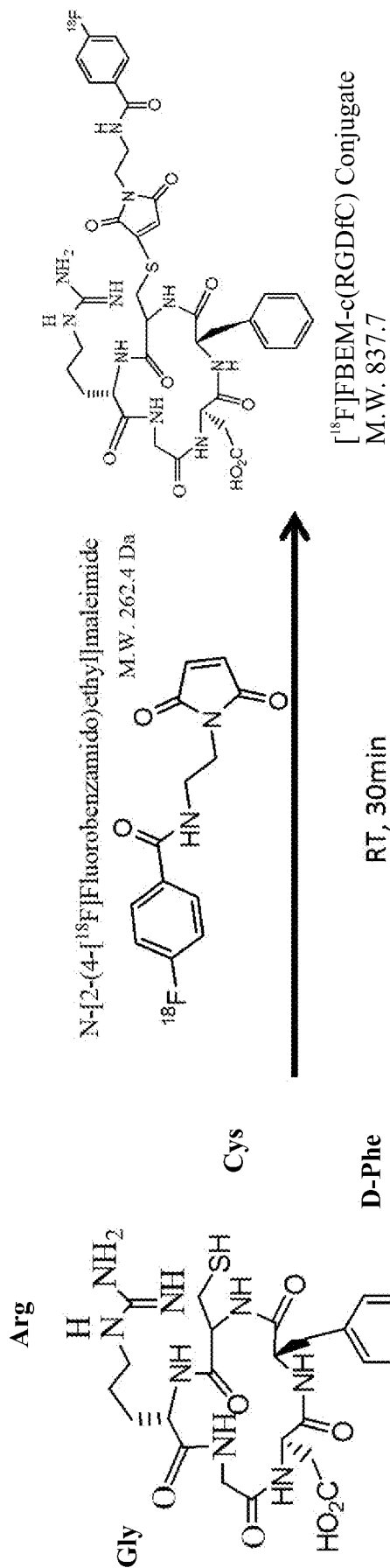


FIG. 6

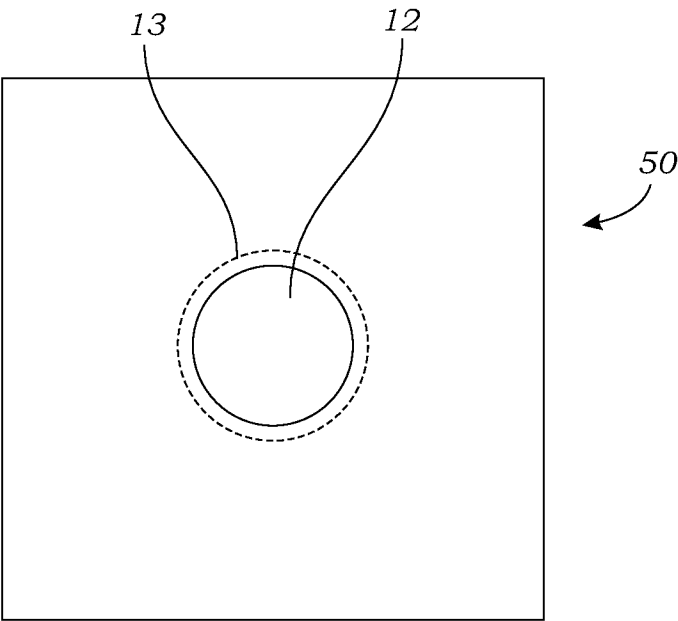


FIG. 7

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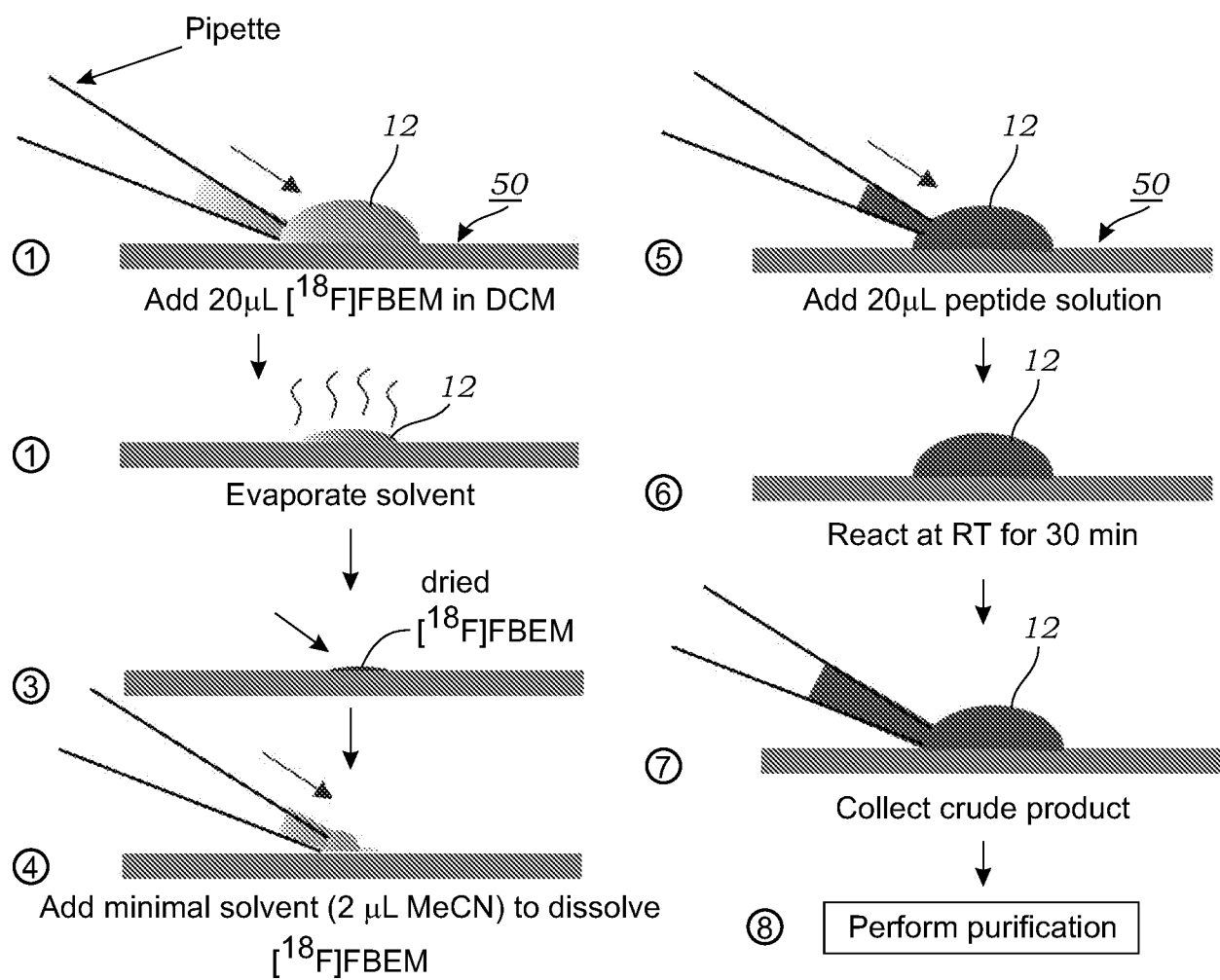


FIG. 8

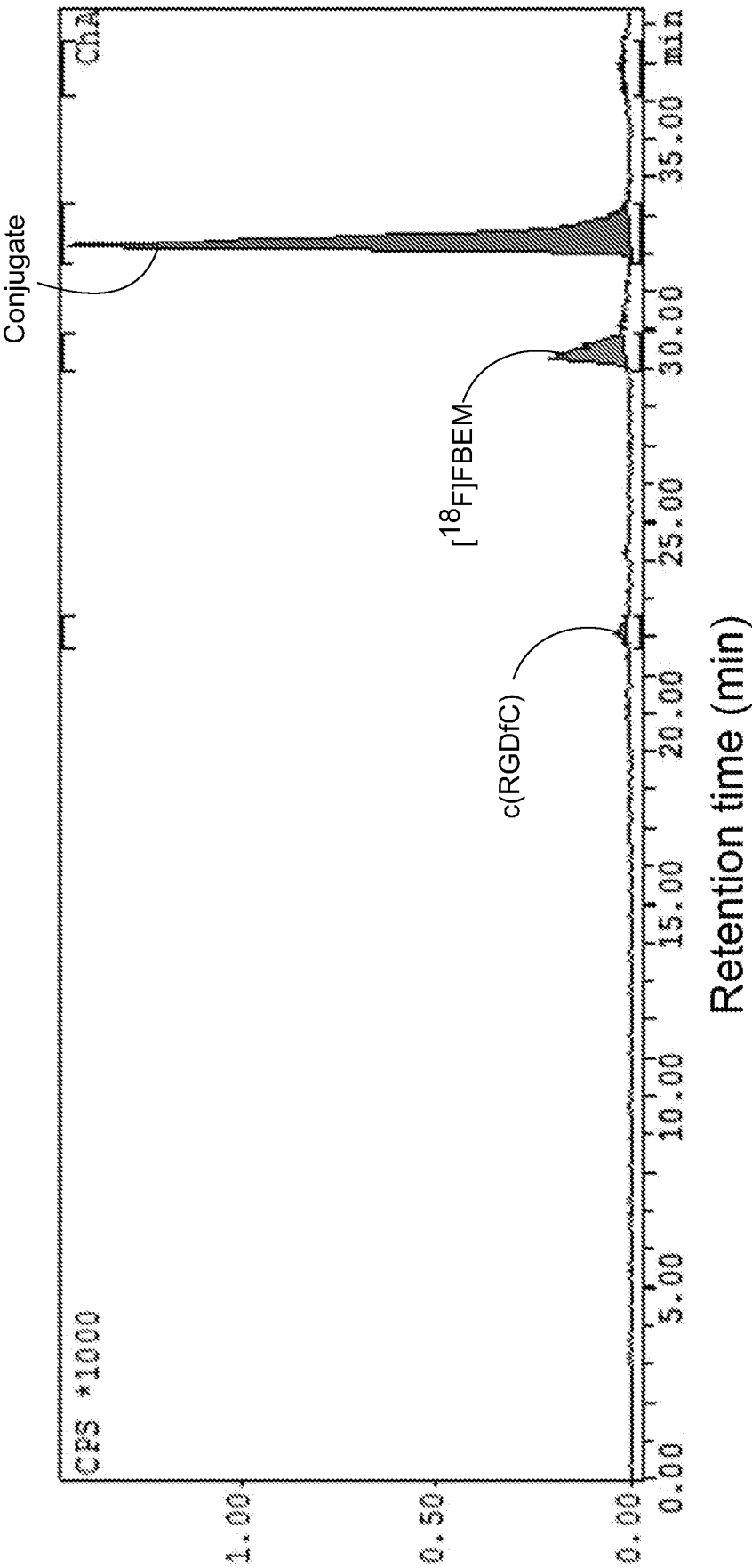


FIG. 9A

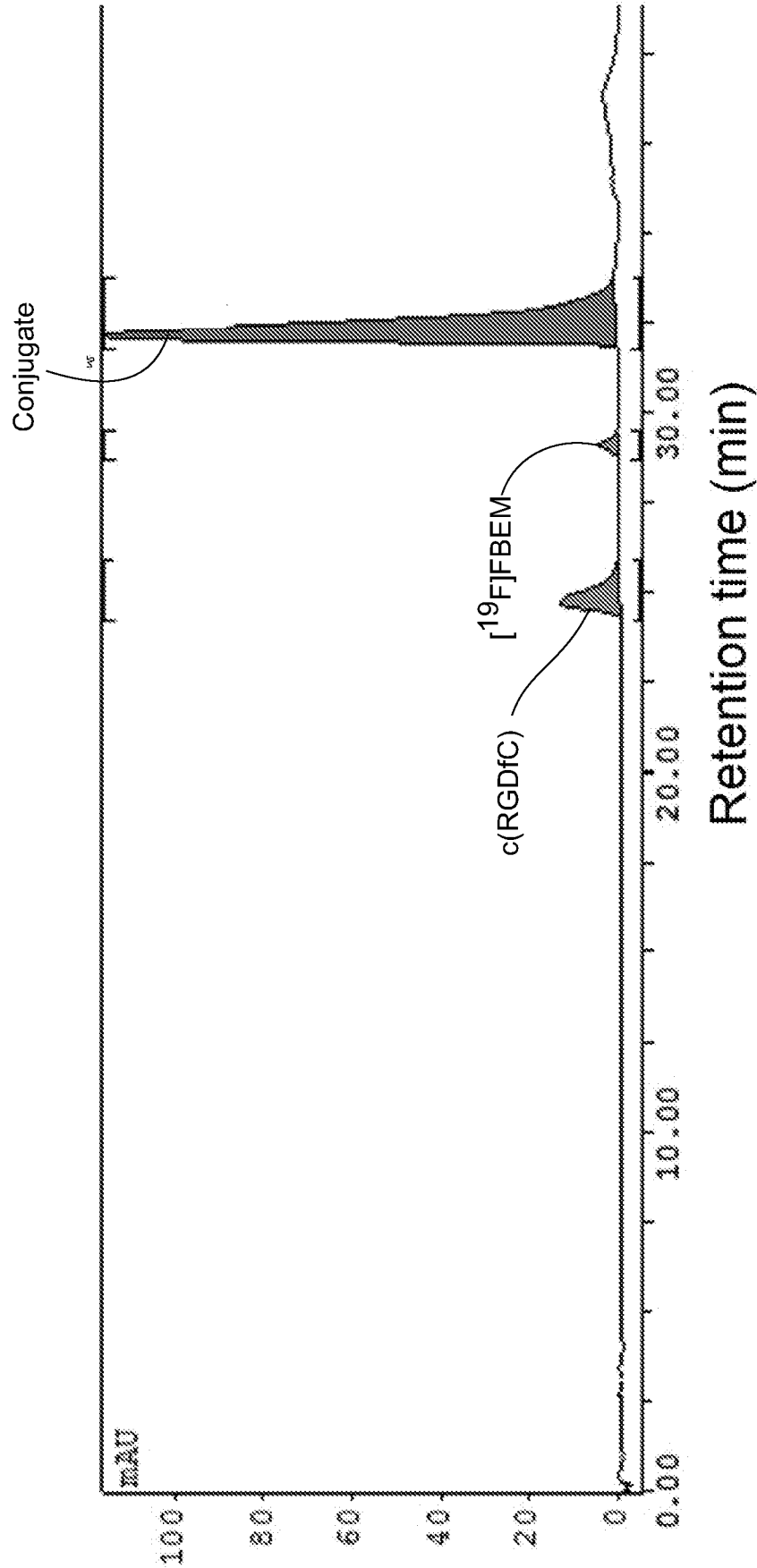
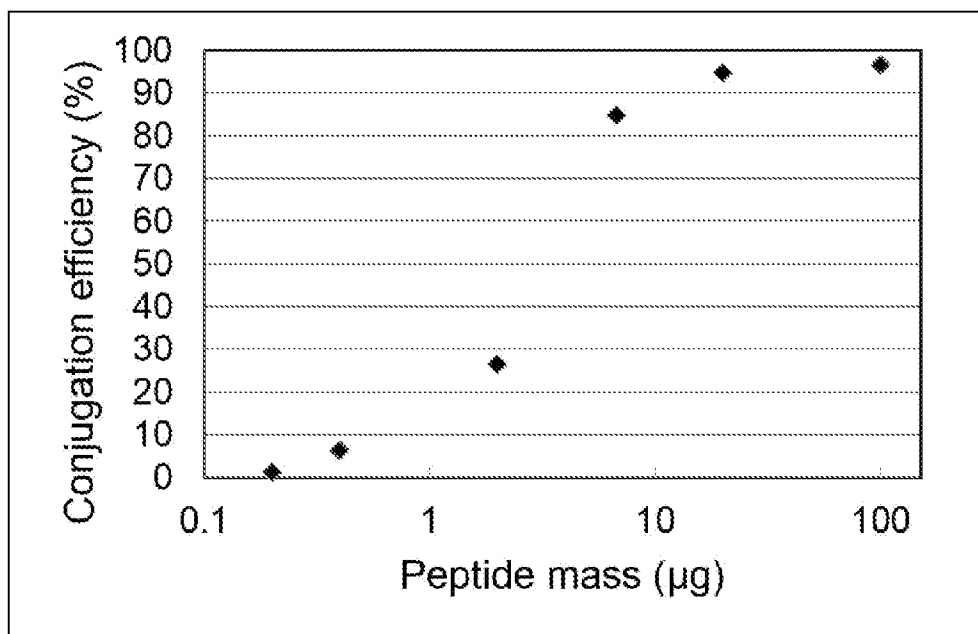
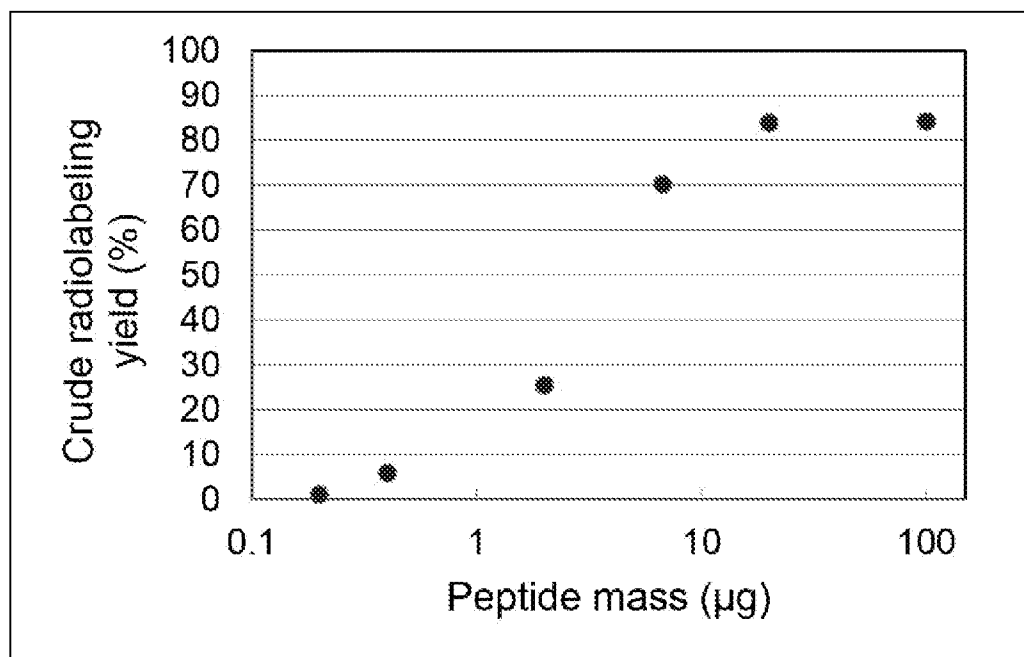
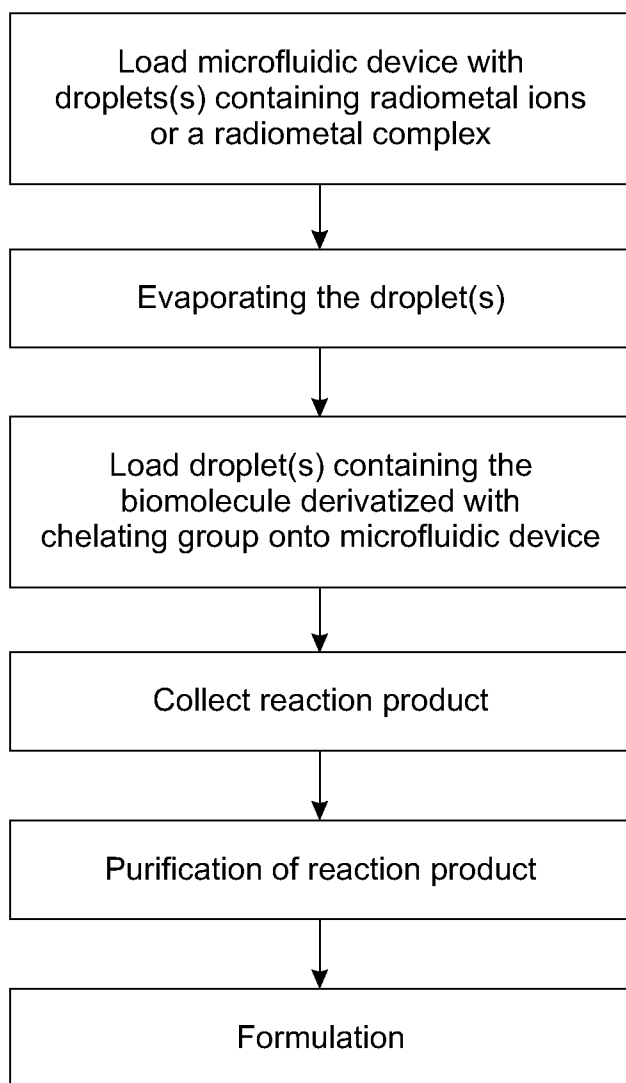


FIG. 9B

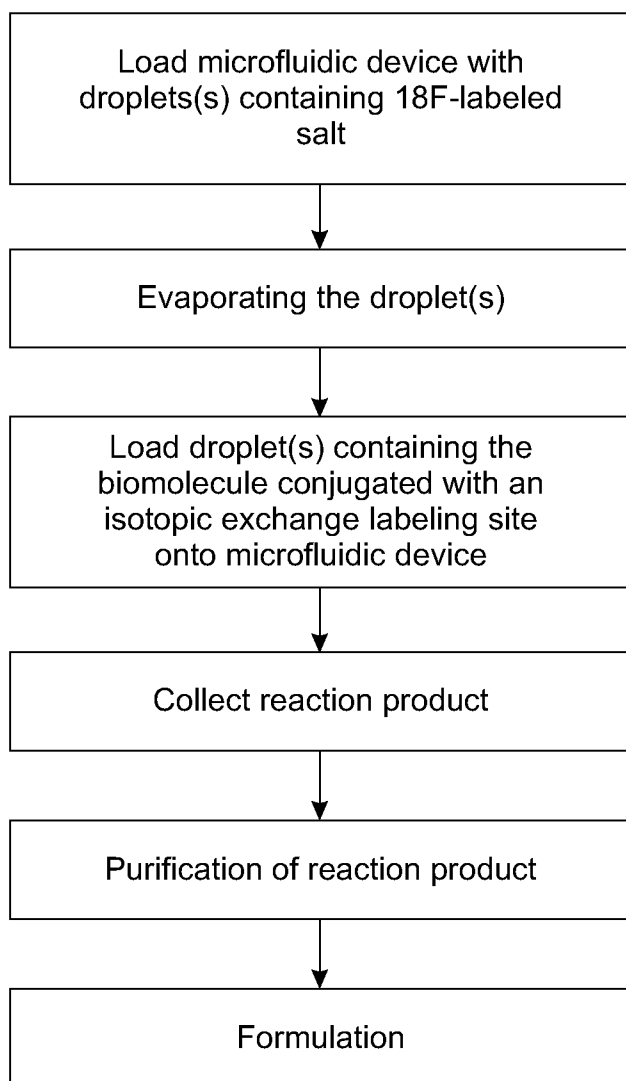
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*FIG. 10**FIG. 11*

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*FIG. 12*

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*FIG. 13*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/050214

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/16; A61K 51/00; A61K 51/04; A61K 51/08; A61K 51/10; C07C 317/08 (2017.01)

CPC - A61K 51/04; A61K 51/0497; A61K 51/082; A61K 51/084; A61K 51/085; A61K 51/088; A61K 51/1045; C07B 39/00; C07B 2200/05; C07C 317/18; C07K 1/13; C07K 7/083; C07K 7/64; C07K 14/31 (2017.08)

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.
X -- Y	US 2016/0107951 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 April 2016 (21.04.2016) entire document	1, 2, 5-8, 10-17 ----- 3, 4, 9, 18-30
Y	WRIGHT et al. "Microfluidic Preparation of a 89Zr-Labeled Trastuzumab Single-Patient Dose," Journal of Nuclear Medicine, 14 January 2016 (14.01.2016), Vol. 57, Pgs. 747-752. entire document	3, 4, 18-23, 26, 30
Y	CAI et al. "A Thiol-Reactive 18F-Labeling Agent, N-[2-(4-18F-fluorobenzamido)ethyl]maleimide, and Synthesis of RGD Peptide-Based Tracer for PET Imaging of avb3 Integrin Expression," Journal of Nuclear Medicine, 01 July 2006 (01.07.2006), Vol. 47, Pgs. 1172-1180. entire document	9
Y	WO 2015/100498 A1 (BRITISH COLUMBIA CANCER AGENCY BRANCH et al) 09 July 2015 (09.07.2015) entire document	24-29
A	US 2010/0254899 A1 (CAPALA et al) 07 October 2010 (07.10.2010) entire document	1-30
A	CHEN et al. "Radiolabelling Diverse Positron Emission Tomography (PET) Tracers Using a Single Digital Microfluidic Reactor Chip," Lab on a Chip, 05 December 2013 (05.12.2013), Vol. 14, Pgs. 902-910. entire document	1-30
A	US 2016/0015838 A1 (UNIVERSITY OF SOUTHERN CALIFORNIA USC STEVENS) 21 January 2016 (21.01.2016) entire document	1-30



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

17 October 2017

Date of mailing of the international search report

07 NOV 2017

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/050214

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2011/0220505 A1 (WANG et al) 15 September 2011 (15.09.2011) entire document	1-30