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(54) **Title: EMULSIFICATION WITH MAGNETIC HYDROGELS**

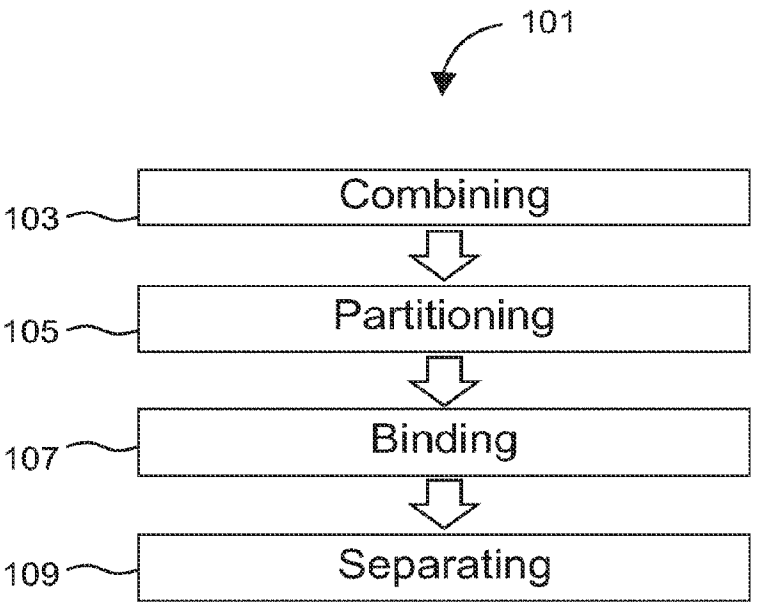

FIG. 1

(57) **Abrégé/Abstract:**
This disclosure relates generally to methods and systems of sample preparation that do not require expensive microfluidic devices or certain time-consuming steps, such as, centrifugation. Instead, systems and methods of the invention use hydrogels that template the formation of partitions inside emulsions and segregate analyte inside the templated partitions. The hydrogels have a hydrogel scaffold embedded with magnetic nanoparticles. The presence of the magnetic nanoparticles provides the ability to interact with and manipulate the templated partitions. Furthermore, the hydrogels include molecular binders that bind with target analyte inside the partitions making the target analyte responsive to magnetic fields for handling analyte during sample preparation.

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(54) Title: EMULSIFICATION WITH MAGNETIC HYDROGELS

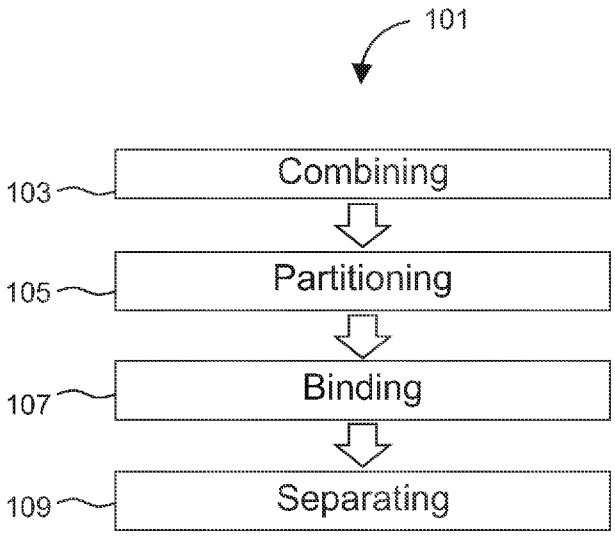

FIG. 1

(57) Abstract: This disclosure relates generally to methods and systems of sample preparation that do not require expensive microfluidic devices or certain time-consuming steps, such as, centrifugation. Instead, systems and methods of the invention use hydrogels that template the formation of partitions inside emulsions and segregate analyte inside the templated partitions. The hydrogels have a hydrogel scaffold embedded with magnetic nanoparticles. The presence of the magnetic nanoparticles provides the ability to interact with and manipulate the templated partitions. Furthermore, the hydrogels include molecular binders that bind with target analyte inside the partitions making the target analyte responsive to magnetic fields for handling analyte during sample preparation.

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EMULSIFICATION WITH MAGNETIC HYDROGELS

Technical Field

This disclosure relates to sample preparation tools that include hydrogels comprising magnetic nanoparticles.

Background

The analysis of single cells results in numerous advantages not available using cells in bulk. Single-cell RNA sequencing, for example, is used to uncover relationships between genetic mutations and disease, identify novel therapeutic agents, and assess the effectiveness of those therapeutic agents in real time. Unfortunately, the wide-spread implementation of such approaches is constrained by the costs associated with isolating single cells and preparing sequencing libraries.

Methods for isolating single cells generally require microfluidic devices that are expensive to use and complicated to operate. Moreover, even once single cells are isolated, the subsequent sequencing reactions require extracted RNA to be purified from crude cell extracts by a long series of centrifugation steps, which is laborious, time-consuming, and often loses valuable material. The current single cell analysis workflow is prohibitively expensive and difficult to access which severely hampers the pace at which new, life-saving discoveries can be made.

Summary

The present invention provides high-throughput systems and methods for sample preparation. The invention provides an emulsion comprising a hydrogel matrix containing magnetic nanoparticles. The emulsion further includes binding elements integrated into the hydrogel matrix. Binding elements are grafted onto the hydrogel matrix and associated with the magnetic nanoparticles. When exposed to a sample, the binding elements bind with their partners (i.e., target analyte). The emulsion forms partitions (e.g., droplets) that sequester analyte. The partitions are manipulated using a magnetic field applied to the vessel containing the emulsion. This allows for the separation of analyte from other, non-analyte material in the sample, with the result being a reduction of background and concentration of the desired analyte. The inclusion of magnetic nanoparticles inside the hydrogel matrix also allows for the manipulation of analyte

during sample preparation even after partitions are broken. Among other things, this allows quick and efficient separation of analyte from crude sample material without centrifugation. As such, systems and methods of the invention reduce costs and time associated with sample preparation while improving sample quality.

Methods of the invention eliminate centrifugation steps during sample preparation. Instead of centrifugation, methods of the invention utilize magnetic fields to manipulate and isolate analyte bound with the hydrogel matrix. The manipulation of analyte is accomplished by applying a magnetic field to the emulsion described above to move and sequester analyte as desired. After that, any sample material that is not attached to a binder can be removed by, for example, pipetting. Thus, methods of the invention are useful for isolating analyte directly from samples that are often difficult to process, such as blood or tissue homogenates. Moreover, because methods and systems of the invention eliminate centrifugation steps, they significantly reduce the time required to prepare samples for analysis and reduce any costs associated with maintaining centrifuge devices. Systems and methods of the invention are particularly well-suited for liquid-handling robotic applications which, due to cost and size constraints, struggle to incorporate centrifuges inside their assemblies.

Methods and systems of the invention can simultaneously segregate analyte into distinct partitions and then process the analyte (e.g., by adding barcodes) inside each of the partitions concurrently within a single reaction tube. In some instances, the target analyte is intracellular. In those instances, cells are combined with the hydrogel emulsion. The emulsion is agitated (e.g., vortexed) to create partitions and single cells are segregated inside the partitions, wherein cells are lysed, and the desired analyte is detected. As such, methods of the invention provide a massively parallel analytical workflow that is inexpensive to use for preparing libraries from single cells.

By isolating analyte in separate partitions and processing the analyte separately inside those partitions, methods of sample preparation avoid cross contamination. Such methods are particularly useful for preparing nucleic acid libraries from single cells and/or amplifying rare nucleic acids while reducing amplification bias. Accordingly, methods include adding barcodes to analyte such that the analyte can be tracked through an assay or procedure. The barcodes are preferably provided via molecular binders (e.g., oligos) that are attached to hydrogels for binding analyte, such that, binding of analyte to the molecular binder effectively results in the analyte

being barcoded. The oligos can include any combination of barcodes but preferably include at least one partition-specific barcode and at least one molecule-specific barcode (e.g., a unique molecular identifier). Thus, upon sequencing, each read can be traced back to a unique molecule in a unique partition.

In one aspect, the disclosure provides methods for sample preparation in which hydrogels (i.e., hydrogel beads) are decorated with molecular binders (e.g., oligos, proteins) and combined in an emulsion with a sample containing analytes to be measured. Each of the hydrogel beads is made of a hydrogel scaffold and contains magnetic nanoparticles. Methods further include partitioning the sample inside the vessel with the hydrogel beads. Partitioning can be achieved by vortexing the vessel and/or by adding partitioning reagents. Upon vortexing, partitions are formed that generally include one or zero hydrogel beads and a single portion of sample containing analyte. Inside the partitions, analyte binds with the molecular binders, thereby tethering the analyte to the hydrogel beads and thus associating analyte with magnetic nanoparticles. Methods further include separating bound analyte from an unbound portion of the sample by applying a magnetic field (which may be as simple as exposing the sample to a magnet in proximity thereto). The unbound portion can be crude sample material, contaminants, or any other undesired portion of sample.

Many analytical processes, such as sequencing, target capture, detection, amplification, etc., require separation of analyte from unwanted material. Methods and systems of the invention provide useful approaches for separating analyte from unwanted material. In preferred embodiments, analyte separation comprises contacting an exterior surface of a vessel or tube containing the sample in emulsion, causing bound analyte to associate near the surface. Methods may further involve pipetting the unbound portion of the sample out from the vessel while contacting the magnet with the surface, or, alternatively, separating bound analyte from the unbound portion of the sample by moving, with the magnet, the bound analyte away from the unbound portion.

The hydrogel emulsions include molecular binders for capturing analyte. The molecular binders can be oligonucleotides, with capture ligands for binding analyte or may be nucleic acid binding proteins, antibodies, or modified nucleic acids (e.g., locked nucleic acids, peptide nucleic acids and the like). The oligos may be linked to the hydrogel beads via acrydite linkages. Because the hydrogel beads contain magnetic nanoparticles, the capture of analyte with a binder

effectively associates the captured analyte with magnetic nanoparticles, and thus enables the captured analyte to be manipulated by applying a magnetic field.

Analytes may be any capture target, including nucleic acid, protein, polysaccharides, and others. In some embodiments, the analyte being characterized is mRNA, which is useful for assessing gene expression. As such, the molecular binders can be oligos with capture ligands, wherein the capture ligands are portions of the oligos comprising sequences of nucleotides complementary to the RNA, e.g., mRNA. For example, the complementary sequences may comprise poly-T sequences for binding poly-A tails of mRNA. Alternatively, the complementary sequences may be sequences complementary to specific mRNAs, such as, mRNA of genes differentially regulated in certain diseases, such as cancer. In other embodiments, the capture ligands may comprise sequences of nucleic acids complementary to portions of DNA. The portions of DNA may be any portion of DNA that is of interest. For example, the capture ligands may be designed to bind with specific portions of DNA associated with certain cancer mutations. In other embodiments, the capture ligands may be designed to bind with target protein. For example, the capture ligands may comprise antibodies. In some embodiments, the beads may comprise a plurality of different types of capture ligands for capturing different types of analyte, such as, capturing at least two of DNA, RNA, and protein.

In preferred instances, each of the molecular binders include a barcode. For example, the molecular binders can include barcodes that are unique to each partition. Such that, each analyte captured inside a partition is barcoded by the partition in which the analyte is captured. The molecular binders may further include barcodes that are unique to each distinct analyte or molecule, e.g., a unique molecular identifier (UMI), so that library preparation yields library members in which analyte, or sequences reads from analyte, contain barcodes specific for each input molecule, and barcode specific for each "partition" (or cell that was isolated in a partition), by virtue of combinations of at least two distinct barcodes.

In some instances, the hydrogel beads further include an agent that improves visibility of the beads inside the vessel. This allows, for example, fluids to be removed during sample preparation from a vessel with increased precision by allowing the researcher or clinician to visualize the hydrogel beads while separating the bound analyte from an unbound portion of sample. The agent may be a dye or a contrasting colored material.

In another aspect, this disclosure provides a reagent for use during sample preparation. The reagent includes a plurality of hydrogel beads. Each one of the hydrogel beads includes a hydrogel scaffold with magnetic nanoparticles embedded therein. Each hydrogel bead further includes molecular binders attached to the hydrogel scaffold. The molecular binders may be covalently attached to the hydrogel scaffold. The molecular binders may include capture ligands for binding nucleic acid or protein. The molecular binders preferably include barcodes. In preferred instances, the molecular binders include at least two distinct barcodes, wherein one barcode is unique to a hydrogel bead, and another barcode is unique to a molecular binder associated with the hydrogel bead. The magnetic nanoparticles contained in the hydrogel scaffold may be ferromagnetic or paramagnetic. Preferably the magnetic nanoparticles are ferromagnetic. The hydrogel beads may be of any size that is sufficient to partition an emulsion and also capture target analyte. As such, in preferred embodiments the beads are at least 10 micrometers in diameter, and preferably greater. For example, the hydrogel beads may comprise a diameter of between 10-200 micrometers. The magnetic nanoparticles are preferably less than 1 micrometer in size, and more preferably, approximately 10 nanometers.

Magnetic particles may be associated with hydrogel beads in a manner that does not compromise partition uniformity, maintains magnetic particle association with each hydrogel, and does not compromise enzyme or reagent function within a partition or within the mixture (e.g., reverse transcription reagents or PCR amplification reagents).

Accordingly, methods of the invention may comprise combining hydrogel beads and magnetic particles in a mixture, thereby associating the magnetic particles with a plurality of the hydrogel beads. The magnetic particles are thereby embedded in the hydrogel bead mixture itself. The hydrogel beads may be designed to associate with the magnetic particles. For example, the hydrogel beads may comprise pores and the magnetic particles may become disposed within the pores of the hydrogel beads. The hydrogel beads and magnetic particles may also be associated using charge interactions between the hydrogel beads and magnetic particles. For example, the hydrogel beads may comprise oligonucleotides disposed on their surface. The oligonucleotides may be designed to carry a charge that attracts the magnetic particles to the hydrogel beads, for example using specific nucleotide modifications. Magnetic particles may be less than about 5 micrometers and also may be silica-coated in order to facilitate association with the hydrogel beads. The association of hydrogel particles and magnetic particles may be

mediated by van der Waals interaction between silica coated magnetic particles and oligonucleotide modifications on the surface of the hydrogel particles.

Once the magnetic particles are associated with the hydrogel beads, the hydrogel beads may be combined, in a vessel, with a sample comprising an analyte. The vessel may be a microcentrifuge tube. The hydrogel beads may then act as template particles to generate a plurality of uniform partitions near-instantly that encapsulate a single one of the hydrogel beads and a portion of the sample to form pre-templated instant partitions (PIPs). The analyte is then bound to the magnetic particles inside the partitions and the bound analyte separated from the sample using a magnet.

Brief Description of Drawings

FIG. 1 diagrams a method of sample prep.

FIG. 2 shows an illustration of a hydrogel bead.

FIG. 3 shows a hydrogel emulsion.

FIG. 4 shows the separation of analyte from a portion of sample inside a vessel.

FIG. 5 illustrates a reagent containing hydrogel beads.

FIG. 6A-6D shows the separation of analyte from a portion of sample.

Detailed Description

This disclosure relates generally to methods and systems for preparing samples for analyte detection. Systems and methods of the invention use hydrogels to create partitions (e.g., droplets) in an emulsion and segregate analyte to be detected in partitions within the emulsion. The hydrogels are made of a polymer matrix embedded with magnetic nanoparticles. The hydrogels include binding elements that specifically bind target analyte. The binding elements are preferably grafted onto the matrix. The binding elements attach the target analyte to the matrix. An applied magnetic field is then used to sequester (e.g., move, capture, or detect) analyte. It is an insight of the invention that the inclusion of the magnetic hydrogels inside templated partitions offers a useful mechanism for manipulating emulsions during sample preparation.

For example, after partitioning, a magnetic field is applied to attract and/or aggregate partitions. Preferably, the partitions are aggregated to a bottom surface of a sample tube. The

aggregation of partitions containing hydrogels at the bottom of the tube displaces any partitions that do not contain hydrogels. Accordingly, any partitions devoid of hydrogels, such as, broken partitions, and any other sample material, such as, excess oil, are displaced towards a top of the sample prep tube. This allows users to separate intact partitions having analyte-binding hydrogels from other unwanted sample material (e.g., broken partitions) by moving the intact partitions with a magnet. The unwanted sample material is easily removed during sample preparation simply by pipetting the material from the sample tube to thereby reduce or eliminate background or contamination.

According to systems and methods of the invention, hydrogels (referred to herein as hydrogel beads) comprise a hydrogel scaffold comprising magnetic inclusions. In particular, the hydrogel scaffold comprises a network of cross-linked polymer chains forming a hydrogel matrix. The magnetic inclusions (e.g., magnetic nanoparticles) are embedded within the hydrogel matrix. In practicing methods of the invention, the hydrogels serve as templates to cause aqueous-in-oil emulsion droplets to form when combined in water with oil and mixed (e.g., vortexed). For example, an aqueous mixture can be prepared in a reaction tube that includes the hydrogels and a sample (e.g., water, saline, buffer, blood, tissue lysate, etc.) having analyte. An oil may be added to the tube, and the tube can be mixed or agitated. The hydrogels act to template the formation of droplets and segregate analyte (e.g., nucleic acid contained in single cells) inside the templated droplets. As such, hydrogels of the invention comprise a size that is sufficient to at least template the formation of a droplet around a cell. For example, the hydrogels may comprise a diameter of at least 10 micrometers, for example, between 10-200 micrometers. Magnetic inclusions contained within the hydrogel scaffold may be substantially smaller, preferably less than one micrometer in diameter, and more preferably approximately 10 nanometers in diameter.

Methods and systems of the disclosure may be conducted with hydrogels by using, for example, the particle-templated emulsification technology described in Hatori et. al., Anal. Chem., 2018 (90):9813-9820, which is incorporated by reference. Essentially, micron-scale hydrogel beads or "template particles" having hydrogel scaffolds containing magnetic inclusions are used to define isolated fluid droplets surrounded by an immiscible partitioning fluid. The hydrogel beads, by virtue of being inside the droplets, allow droplets and analyte to be easily handled during sample preparation with a magnetic field.

FIG. 1 diagrams a method 101 of sample preparation. The method 101 includes combining 103 hydrogel beads with sample comprising analyte inside a vessel. The hydrogel beads comprise a hydrogel scaffold embedded with magnetic inclusions (e.g., magnetic nanoparticles). The beads also include molecular binders. Once combined 103, the method 101 involves partitioning 105 the sample inside the vessel. After partitioning 105, the methods include binding 107 analyte to the molecular binders inside the partitions, and separating 109, with a magnet, bound analyte from an unbound portion of the sample.

While the hydrogel beads and sample may be combined 103 or added in any order to a vessel, it may be useful to provide the vessel with the hydrogel beads included therein, and to add the sample comprising analyte directly onto the hydrogel beads. For example, the hydrogel beads may be manufactured as a custom-made reagent provided to a researcher or clinician inside the vessel for receiving the sample directly therein.

As such, in some embodiments the hydrogel beads are provided in the vessel for performing steps of the method 101. Any suitable vessel may be used. For example, a sample vessel may be, for example, a 0.5 to 1.5 milliliter microcentrifuge tube, such as those sold under the trademark EPPENDORF. The sample vessel may be a blood collection tube such as the collection tube sold under the trademark VACUTAINER. The tube may be a conical centrifuge tube sold under the trademark FALCON by Corning Life Science. In preferred embodiments of the method, the hydrogel beads are provided in the vessel within an aqueous media such as a buffer, nutrient broth, saline, or water.

The sample may be added directly into the vessel, e.g., directly upon obtaining the sample or after some minimal sample preparation step. The sample that contains the analyte may be from any biological source. Suitable samples include environmental, clinical, library specimen, or other samples with known or unknown analyte present. Suitable samples may include whole or parts of blood, plasma, cerebrospinal fluid, saliva, tissue aspirate, microbial culture, uncultured microorganisms, swabs, or any other suitable sample. For example, in some embodiments, a blood sample is obtained (e.g., by phlebotomy) in a clinical setting. Whole blood may be used, or the blood may be spun down to isolate a component of interest from the blood, such as peripheral blood monocytes (PBMCs). Preferably an oil is added to the tube (which will typically initially overlay the aqueous mixture). A surfactant may also be added as discussed further below.

The analyte can be any chemical species, substance, or chemical constituent that is of interest. In some embodiments, the analyte comprises DNA. For example, analyte may comprise genomic DNA taken from a nucleus of a cell. In other instances, the DNA may be cell free DNA, such as, circulating tumor DNA, cell free mitochondrial DNA, or cell free fetal DNA. For example, the DNA may comprise cell free DNA that is present at elevated levels in blood in a subject having cancer, such as, breast cancer.

In some instances, the analyte comprises RNA. The RNA may comprise one or more of messenger RNA, transfer RNA, ribosomal RNA, micro RNA, or the like. The RNA may be isolated from a cell or extracellular vesicle.

In some instances, the target analyte comprises protein or protein fragments. The analyte may comprise, for example, a chain of amino acids that code for a portion of a protein.

The method 101 further includes partitioning 105 the sample inside the vessel with the hydrogel beads. Partitioning 105, in a general sense, involves the action of dividing the combined mixture of beads and sample into parts. The parts, i.e., partitions, preferably comprise aqueous droplets that are substantially monodispersed within the vessel. The partitions (i.e., droplets) are all formed at the moment of vortexing, essentially instantly, as compared to the formation of droplets by flowing two fluids through a junction on a microfluidic chip, which is limited by time as each droplet must be formed separately. Each droplet thus provides an aqueous partition, surrounded by oil.

Partitioning 105 can be performed by agitating the vessel containing the sample, hydrogel beads, and oil combined therein. Upon agitating, the hydrogel beads serve as “templates” while the shear forces generated from agitating the vessel causes the formation of water-in-oil partitions with, ideally, a single hydrogel bead and a portion of sample comprising analyte inside each partition. Agitating can be performed by pipetting the mixture to shear the fluid causing partitioning 105. Alternatively, partitioning may be performed by contacting the vessel with a standard lab-bench vortexer. It may be found that during the vortexing, the mixture partitions into the aqueous droplets within about 5 to about 50 seconds, resulting in sample comprising analyte to be segregated inside said partitions with the hydrogel beads. In yet other embodiments, partitioning 105 may be performed by adding reagents that cause the mixture to shear.

Upon partitioning 105, a substantial portion of the resultant droplets will contain a single hydrogel bead and a portion of sample comprising analyte. Partitions formed according to

methods of the disclosure are generally monodisperse, meaning that the vast majority of the droplets will include one hydrogel bead and the vast majority of hydrogel beads will template into one partition, i.e., droplet. Said another way, monodisperse means that comparing the number of hydrogel beads initially provided in the aqueous mixture to the number of droplets produced by vortexing, the smaller number will be at least 90% of the larger number, and in practice usually at least 95%, more preferably 98% or 99%.

Partitions containing more than one or zero beads can be removed, destroyed, or otherwise ignored. For example, in preferred embodiments, methods of the invention include manipulating the emulsion by interacting with templated partitions (i.e., those that were templated by hydrogel beads). For example, in some embodiments, a magnet can be used to interact directly with intact, templated partitions to aggregate or pellet those partitions containing hydrogel beads to a bottom of the vessel. Partitions devoid of hydrogel beads are consequently pushed upwards towards the opening of the vessel. A pipette can then easily be inserted into the vessel to remove or destroy partitions lacking a hydrogel. Advantageously, by removing partitions devoid of hydrogel beads, background noise produced during sequencing reactions is substantially reduced.

In some embodiments, analyte is segregated into the partitions as single cells. In such embodiments, the single cells are lysed to thereby release the contents of the single cells, including analyte, inside the partitions. An important insight of the disclosure is that the hydrogel beads may themselves contain reagents that promote useful reactions inside the partitions, such as cell lysis. For example, reagents, detergents, enzymes, and cations that induce cell lysis may be provided by the hydrogel beads. Such material may be provided by the hydrogel beads via internal compartments inside the hydrogel. In some embodiments, lysing may involve heating the partitions to a temperature sufficient to release lytic reagents, such as, divalent cations, contained inside the hydrogels into the partitions. Lysing may be accomplished using mechanical, chemical, or enzymatic means, the addition of heat, divalent cations (e.g., Mn2+ and/or Mg2+), or any combination thereof.

Each of the hydrogel beads may include a plurality of molecular binders for binding analyte. The hydrogel beads may include hundreds to thousands to millions of distinct molecular binders for binding analyte. The molecular binders may be designed to bind with the identical analyte, for example, gene transcripts of an identical gene, or the molecular binders may be

designed to bind with distinct analyte, such as, gene transcripts coded by different genes. In some instances, the molecular binders may be made to bind with different types of analyte (i.e., DNA, RNA, or protein). For example, each hydrogel bead may include molecular binders that capture more than of DNA, RNA, or protein. Accordingly, methods and systems of the invention can provide for single-cell analysis, including single-cell multi-omic analysis, of the transcriptome, proteome, and genetic material of single cells, which has obvious advantages of thorough, efficient, full-characterization of cell function.

Preferably, the molecular binders are grafted onto the hydrogel scaffolds of the hydrogel beads. The molecular binders may be any biological material with an attractive force towards the target analyte. Preferably, the molecular binders are oligos, also known as oligonucleotides, which comprise contiguous strings of nucleic acids. At least a portion of the oligos include a capture ligand. The capture ligand is the portion of the molecular binder, e.g., oligo, which is made to have an affinity for target analyte, such as, by having a complementary nucleotide sequence.

After sample comprising analyte has been segregated into the partitions, target analyte binds with the molecular binders inside the partitions. In some embodiments, target analyte may comprise DNA, and portions of the molecular binders may comprise sequences that are complementary with portions of the target DNA. Binding of analyte with molecular binders inside the partitions may occur via Watson-Crick base pairing resulting in hybridization of the analyte with portions of the molecular binders. As such, methods may include incubating a vessel at a temperature for a period of time sufficient for hybridization to occur. While the exact temperatures and time periods will vary depending on specific sequence compositions of analyte and binders, it may be found that incubating the vessel at 37 degrees Celsius for 1 hour is sufficient for the target analyte to bind with the molecular binders.

In some instances, the target analyte is RNA. As such, the molecular binders may comprise oligos comprising base pair sequences that are complementary to target RNA. The target RNA may comprise a subset of RNA released by a single cell. For example, the subset of RNA may be genes known to be differentially regulated during disease or pathogenic infection. The subset of RNA can be bound with hydrogel beads on account of a portion of the molecular binders comprising sequences complementary to at least a portion of the subset of RNA. In some instances, it may be desirable to profile total messenger RNA released by a single cell. In such

instances, each hydrogel bead may include molecular binders, wherein at least a portion of the binders comprise a poly-T sequences for binding with poly-A tails of the messenger RNA.

In some instances, the target analyte is protein. Where the target analyte is protein, molecular binders of the invention may comprise oligos with an antibody or a portion of an antibody attached thereto. The antibody may be attached to oligos via methods known in the art, such as those discussed in Wiener, 2020, Preparation of single- and double-oligonucleotide antibody conjugates and their application for protein analytics, Scientific Reports 10 (1457), which is incorporated herein by reference.

Methods of the invention involve separating bound analyte from an unbound portion of the sample by applying a magnetic field. The magnetic field may be applied with a magnet. The magnet can be any material or object that produces a magnetic field. Because analyte is bound with hydrogel beads containing magnetic nanoparticles (e.g., ferromagnetic nanoparticles) analyte can be separated from the rest of the sample with a magnet. For example, separating may comprise contacting the magnet to an exterior surface of the vessel thereby causing the magnetic nanoparticles embedded inside hydrogel beads, and consequently, bead-bound analyte, to associate with a surface inside the vessel that is adjacent the surface on which the magnet is positioned. Separating may further include pipetting any unbound portion of the sample from the vessel while contacting the magnet with the surface of the vessel to thereby separate the unbound portion of the sample from the bound analyte. Alternatively, separating bound analyte may involve moving, with the magnet, the bound analyte away from the unbound portion of the sample.

FIG. 2 shows an illustration of a hydrogel bead 201 with magnetic nanoparticles 207. In particular, illustrated is a hydrogel bead 201 comprising a hydrogel scaffold 203 with magnetic nanoparticles 207 embedded within the scaffold. The hydrogel scaffold 203 comprises a hydrogel polymer. The hydrogel polymer can be any suitable material, such as, for example, polyacrylamide (PAA), bis-acrylamide, agarose, poly-ethylene-glycol (PEG), or polystyrene.

In some embodiments, the hydrogel beads 201 are premade and purchased from a vendor. In some embodiments, the hydrogel beads 201 are made inhouse. The scaffolds 203 of the hydrogel beads 201 may comprise, for example, 6.2% acrylamide (Sigma-Aldrich), 0.18% N,N′-methylene-bis-acrylamide (Sigma-Aldrich), and 0.3% ammonium persulfate (Sigma-Aldrich), which are used for PAA scaffold generation. A total of 14% (w/v) 8-arm PEG SH (Creative

PEGworks) in 100 mM NaHCO3 and PEGDA (6 kDa, Creative PEGworks) in 100 mM NaHCO3 may be used for PEG scaffold generation. Or, a 1% low melting temperature agarose (Sigma-Aldrich), which may be used for agarose scaffold generation.

Agarose and PEG scaffold solutions may be injected into a droplet generation device with oil (HFE-7500 fluorinated oil supplemented with 5% (w/w) deprotonated Krytox 157 FSH) using syringe pumps (New Era, NE-501). The PAA scaffold solution may be injected into the droplet generation device with the fluorinated oil supplemented with 1% TEMED. The hydrogel solution and oil are preferably loaded into separate 1 mL syringes (BD) and injected at 300 and 500 microliters, respectively, into the droplet generation device using syringe pumps.

A desired quantity of magnetic nanoparticles can be added into the droplet generation device to thereby create hydrogel with magnetic particles embedded therein. The magnetic nanoparticles are preferably ferromagnetic nanoparticles, e.g., iron or iron oxide, for example, such as the magnetic particles sold under the trade name Ferrotec. The hydrogel beads may be made to contain a magnetite nanoparticle content with as high as 30% by weight or more.

To improve biocompatibility, it may be helpful to coat the magnetic nanoparticles with a synthetic or biological polymer before adding the magnetic nanoparticles to the droplet generation device. The magnetic nanoparticles may be coated with, for example, PEG. The PAA and PEG droplets are collected and incubated for 1 hour at room temperature for gelation. The agarose droplets are incubated on ice for gelation. After gelation, the gelled droplets are transferred to an aqueous carrier by destabilizing them in oil with the addition of an equal volume of 20% (v/v) perfluoro-1-octanol in HFE-7500. The particles are washed twice with hexane containing 2% Span-80 (Sigma-Aldrich) to remove residual oil. Following the hexane wash, the particles are washed with sterile water until all oil is removed.

For further discussion on making hydrogel beads according to aspects of the invention, see Berensmeier, 2006, Magnetic particles for the separation and purification of nucleic acids, Appl Microbiol Biotechnol, 73(3): 495–504; Philippova, 2011, Magnetic polymer beads: Recent trends and developments in synthetic design and applications, European Polymer Journal, 47 (4): 542-559; Suh, 2012, Synthesis of magnetic hydrogel microparticles for bioassays and tweezer manipulation in microwells, Microfluidics and Nanofluidics, 13: 665–674, Bong, 2011, Magnetic Barcoded Hydrogel Microparticles for Multiplexed Detection, Langmuir 26(11): 8008–8014, each of which is incorporated herein by reference.

Preferably, the hydrogel scaffold 203 includes a concentration of hydrogel that effectively prevents the magnetic nanoparticles from seeping from the hydrogel beads. The concentration of the hydrogel may be about 0.009 – 1.0 percent hydrogel, and more preferably, about 0.01-0.5 percent hydrogel.

In some embodiments, the hydrogels may be disulfide soluble hydrogels. Disulfide soluble hydrogels can allow for release of functional hydrogels after partitioning.

The hydrogel beads 201 further include molecular binders 209 for binding analyte. Any number of molecular binders 209 may be included. The molecular binders 209 may be designed to bind with the identical analyte, for example, gene transcripts of an identical gene, or the molecular binders may be designed to bind with distinct analyte, such as, gene transcripts coded by different genes. In some instances, the molecular binders may be made to bind with different types of analyte (i.e., DNA, RNA, or protein). Accordingly, hydrogel beads 201 of the invention can be used for single-cell analysis, including single-cell multi-omic analysis, to study the transcriptome, proteome, and genome of single cells.

The molecular binders 209 are preferably oligos. The oligos may comprise capture ligands for binding target analyte. For example, at least a portion of the oligo may comprise a sequence of nucleotides that is complementary to at least a portion of target analyte. The molecular binders 209 are preferably attached to an exterior surface of the hydrogel scaffold 203. In some instances, the molecular binders are attached to an exterior surface of the hydrogel scaffold via acrydite linkages.

In some instances, the hydrogel bead 201 may include an agent that improves visibility of the bead 201, for example, when the bead is being used during sample preparation. The agent may be any natural or synthetic substance that, when added to the hydrogel, changes the color of the hydrogel. By changing the color, the hydrogel beads may be easier to see inside the vessel. The advantage of improved visibility is that it makes it easier to separate hydrogel beads having bound analyte away from any other portion of the sample since the researcher or clinician can observe, in real time, whether material that is being moved away from a tube includes the beads based on the visualization of the agent, such as a dye.

Because methods of the disclosure are useful for isolating analyte into partitions, and then preparing libraries of large numbers of molecules in each partition, some methods of the invention include barcoding analyte such that the analyte can be tracked through an assay.

Accordingly, aspects of the invention provide reliable methods for "barcoding" analyte inside partitions. The barcodes are preferably provided by the molecular binders, such that, binding of analyte to the molecular binder effectively results in the analyte being barcoded. The term barcode should be understood to mean any number of barcodes, index or index sequence, or UMIs, which are unique, i.e., distinguishable from other barcodes. The sequences may be of any suitable length which is sufficient to distinguish the barcode, or index, sequence from other barcode sequences. A barcode, or index, sequence may have a length of 4,5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 nucleotides, or more.

Specifically, each of the molecular binders are preferably designed to include one or more distinct barcodes. Preferably, the one or more barcodes include at least one partition-specific barcode (i.e., a barcode that is unique to each hydrogel bead) and an analyte specific barcode (i.e., a barcode that is unique to each molecule, e.g., a unique molecular identifier (UMI). The partition-specific barcode is advantageous because, for example, during single cell analysis, a researcher or clinician can determine which analytes were contained together within a single cell based on the presence of identical barcodes.

UMIs are a type of barcode that may be provided to a sample to make each nucleic acid molecule, together with its barcode, unique, or nearly unique. This may be accomplished by adding one or more UMIs to one or more molecular binders of the present invention. By selecting an appropriate number of UMIs, every molecule of analyte in the sample, together with its UMI, will be unique or nearly unique.

UMIs are advantageous in that they can be used to count transcripts in a sample from sequence data. Because each transcript is tagged with an essentially unique UMI barcode, sequencing the transcripts will create sequence read data that is unique for each transcript. This is valuable when multiple transcripts present in a cell have identical sequences. Attaching a UMI to each allows one to count the number of identical transcripts in the original cell (regardless of the presence of other barcode information such a cellular barcodes and/or sequencing instrument index sequences). When a transcript with an attached UMI is amplified and then sequenced, the resultant sequence read data will have an arbitrary number of identical sequence reads, produced by sequencing clonal amplicons of the transcript created during amplification. To count the transcripts present in the sample, one "de-duplicates" the sequence read data with the UMI sequences included (sometimes called collapsing reads) and counts the remaining unique

sequences. Transcript counting may be performed with existing software tools for deduplicating reads based on UMIs. See Islam, 2014, Quantitative single-cell RNA-seq with unique molecular identifiers, Nat Meth 11(2):163-6 and Liu, 2019, Algorithms for efficiently collapsing reads with Unique Molecular Identifiers, Peer J 7:e8275, both incorporated by reference.

UMIs are advantageous in that they can be used to correct for errors created during amplification, such as amplification bias or incorrect base pairing during amplification. For example, when using UMIs, because every nucleic acid molecule in a sample together with its UMI or UMIs is unique or nearly unique, after amplification and sequencing, molecules with identical sequences may be considered to refer to the same starting nucleic acid molecule, thereby reducing amplification bias. Methods for error correction using UMIs are described in Karlsson et al., 2016, Counting Molecules in cell-free DNA and single cells RNA", Karolinska Institutet, Stockholm Sweden, incorporated herein by reference.

FIG. 3 shows a hydrogel emulsion. In particular, shown is a vessel 301 containing sample 303 and hydrogel beads 307 combined inside an emulsion. Upon mixing the emulsion, the hydrogel beads 307 template partitions and segregate analyte therein. Any templated partition includes one of the hydrogel beads 307. The partitions containing the hydrogel beads 307 can be manipulated, for example, moved within the vessel, by applying a magnetic field to the vessel on account of the magnetic nanoparticles contained within the hydrogel.

FIG. 4 shows the separation of analyte from a portion of sample 409 inside a vessel 301. In particular, the figure shows hydrogel beads 307 bound with analyte being separated from a portion of sample 409 using a magnet 411. The magnet 411 is attached to a magnetic station 413 for holding the vessel during sample preparation. Specifically, once the vessel is inserted into the magnetic station 413, the presence of the magnet 411 near a side of the vessel 301 causes magnetic nanoparticles embedded in the hydrogel beads 307 to move towards the side of the vessel 301 nearest the magnet 411. As such, hydrogel beads 307 bound with analyte are aggregated inside the vessel 301. Once the aggregated, the portion of the sample 409 not comprising the hydrogel beads can be easily and effectively removed from the vessel 301 using a pipette, or by dumping the contents of the vessel, without centrifugation.

FIG. 5 illustrates a reagent 501 comprising hydrogel beads 503. The hydrogel beads 503 may be provided in an aqueous solution (e.g., water, saline, or buffer) or may be provided in dried format. Making reference to FIG. 2, each of the hydrogel beads is preferably made of a

hydrogel scaffold with magnetic nanoparticles embedded inside the scaffold. The hydrogel beads 503 may further include molecular binders (e.g., oligos). The molecular binders are preferably grafted onto the hydrogel scaffold. The molecular binders may include capture ligands for binding one of nucleic acid or protein. The capture ligands are preferably portions of the oligos that code for nucleic acid sequences that are complementary to target analyte. The nucleic acid sequences can be designed using online tools that are well known in the art, for example, as described in Jayaraman, 2019, AnthOligo: Automating the design of oligonucleotides for capture/enrichment technologies, bioRxiv, the contents of which are incorporated by reference.

The molecular binders are made to include one or more barcodes. Preferably, the one or more barcodes include at least one barcode that is unique to all the binders attached to a particular hydrogel bead. The one or more barcodes further include an analyte specific barcode, i.e., a barcode that is unique to each molecule, e.g., a UMI. The hydrogel-specific barcode is advantageous because, for example, during single cell analysis, a researcher or clinician can determine which analytes were contained together within a particular partition, and thus, from a single cell based on the presence of identical barcodes.

According to some aspects of the invention, this disclosure provides methods for preparing libraries from single cells. The libraries may be used for sequencing by next-generation sequencing devices. The hydrogel beads embedded with magnetic nanoparticles may be combined with aqueous liquid and cells and other reagents are introduced (reagents, such as lysis reagents, may be delivered within the hydrogel beads). An oil is overlaid, optionally with a surfactant (discussed in greater detail above), and the mixture is sheared or vortexed, which causes the beads to act as templates to form monodisperse emulsions, which may be referred to as pre-templated instant partitions, or “PIPs”. In general, each partition includes one or zero hydrogel beads, sometimes referred to as a template particle, and a volume of partitioned fluid, and a surfactant stabilized shell or surface. To reduce background, it may be desirable to remove or destroy partitions devoid of hydrogel.

Cells may be lysed inside partitions to release target analyte for binding with binders. In some instances, lysis reagents may diffuse from the hydrogels into the aqueous partitions of the emulsion. In some embodiments, nucleic acids are fragmented to create diversity within the pool of nucleic acids so as to uniquely identify analyte without using UMIs, for example, as described in co-pending Application No. 63/109,035, which is incorporated herein by reference.

After lysis, analyte, e.g., RNA that is expelled by the single cells binds with molecular binders inside the partitions. Preferably, the molecular binders are attached, via covalent bonds, to a surface of the hydrogel, thereby tethering the RNA to the hydrogel beads. However, in some instances, it may be advantageous to package the molecular binders within a compartment of the hydrogels and release the molecular binders from the hydrogels via an external stimulus (e.g., heat), inside the droplets. It may be found that by releasing the binders from an internal compartment that certain undesirable intra- or inter-molecular binder interactions occurring during binding of analyte, such as, RNA to the binders may be avoided. In other embodiments, it is preferable to have the molecular binders attached to the hydrogels so as to allow for precise handling of analyte during sample preparation by magnetism after rupturing the partitions.

In some embodiments, a poly-T end of the molecular binder hybridizes to and captures mRNA via a poly-A tail of the mRNA. After binding the mRNA with the molecular binder, the partitions may be broken. Any emulsions can be freely broken, and products pooled due to the barcodes provided by the molecular binders from the hydrogel beads.

After the emulsions are broken, a magnet may be contacted with the vessel to pellet hydrogels inside the vessel. One or more wash steps may be used to rid the sample of unwanted cell debris and any other contaminants. After which, reverse transcription (RT) may be performed. The reverse transcriptase copies the bound mRNA into complementary cDNA. In some embodiments, the reverse transcriptase adds untemplated C bases during RT. Preferably, the oligos are attached to beads and after RT each extends to include a cDNA sequence followed by several terminal C bases. A template switching oligo (TSO) may be introduced and hybridized to the Cs. The TSO can be used to add a common sequence to the cDNA that is used downstream for library creation. Polymerase copies the TSO thereby extending the oligos on the bead. The TSO may include a preferred sequencing adaptor, such as, the Illumina P5 adaptor. The final product may optionally include indexed sequencing adaptors and may be amplified using, for example, known platform-specific sequencing amplification primers such as Illumina forward and reverse primers.

Sequencing yields genetic sequences that can be de-multiplexed informatically by referencing the information introduced by the ligation barcodes. Embodiments of the ligated barcodes of this disclosure are useful in methods for reverse transcribing mRNA into complementary DNA (cDNA) from cells isolated within aqueous partitions.

In certain aspects, the disclosure provides a library preparation method for RNA-sequencing. The method includes preparing a mixture that includes cells and reagents for reverse transcription (RT) and vortexing or optionally pipetting the mixture. During the vortexing (or pipetting), the mixture partitions into aqueous-in-oil droplets that each essentially include zero or one cell, the cells are lysed to release mRNA into the droplets, and reverse transcriptase copies the mRNA into cDNAs. The method preferably further includes amplifying the cDNAs into a library of amplicons. Preferably the mixture includes beads that template the formation of the droplets upon vortexing. The beads may be gels that include paramagnetic or preferably ferromagnetic nanoparticles therein. The mixture may be aqueous and the method may include adding an oil onto the mixture prior to the vortexing/pipetting. The method may include heating the mixture to a temperature that promotes activity of the reverse transcriptase (e.g., between about forty and about fifty degrees C). The mixture is preferably sheared by any suitable mechanism or device, such as a benchtop vortexer or shaker, a pipette (e.g., micropipette), a magnetic or other stirrer or similar. The beads may be linked to molecular binders, sometimes referred to as capture oligos, that have a free, 3' poly-T region. The beads may also include cDNA capture oligos that have 3' portions that hybridize to cDNA copies of the mRNA. The 3' portions of the cDNA capture oligos may include gene-specific sequences or oligomers. The oligomers may be random or "not-so-random" (NSR) oligomers (NSROs), such as random hexamers or NSR hexamers. The beads may be linked to capture oligos that include one or more handles such as primer binding sequences cognate to PCR primers that are used in the amplification step or the sequences of NGS sequencing adaptors. The cDNA capture oligos may include template switching oligos (TSOs), which may include poly-G sequences that hybridize to and capture poly-C segments added during reverse transcription.

In some embodiments, emulsions of the invention include one or more surfactants. Inclusion of a surfactant may improve stability of the emulsion. Exemplary surfactants are described in published application WO2020069298A1, which is incorporated by reference.

Because emulsions generated by systems and methods of the invention allow users to interact with intact partitions during sample preparation, for example, by moving intact partitions within a tube and separating intact partitions from broken partitions, these methods are particularly well suited for droplet PCR applications (dPCR) to segregate target analyte (i.e.,

nucleic acids) from unwanted material and directly quantify and clonally amplify the target analyte.

For example, the method may involve partitioning a PCR solution containing hydrogel and analyte into tens of thousands of nano-liter sized droplets. The solution may include components of a TaqMan assay, e.g., fluorescence-quencher probes, primers, a PCR master mix (DNA polymerase, dNTPs, MgCl2), and reaction buffers at optimal concentrations. The PCR solution can be divided into smaller reactions and then undergo PCR individually. After multiple PCR amplification cycles, the samples are checked for fluorescence with a binary readout of "0" or "1". The fraction of fluorescent droplets can be recorded and used to quantify nucleic acid. According to aspects of the invention, the partitions can be manipulated, e.g., moved, sorted, captured, etc., based on the presence of magnetic nanoparticles incorporated inside hydrogel. In some instances, this is useful for accurate and efficient quantification of droplets using fluorescence because magnets can be used to separate intact partitions from broken partitions before quantification of fluorescence to improve background signal and generate more reliable data.

Hydrogels embedded with magnetic nanoparticles offer precise methods for interacting with both analyte and intact partitions during sample prep. They also eliminate the need for centrifugation steps, which are required for the isolation and purification of analyte by prior art methods. The precision of these methods, and the replacement of centrifuges, makes them well suited for automatic sample preparation applications. For example, robotic devices can be made that prepare analyte for analysis by separating analyte from other portions of sample using magnetic stations. The magnetic stations may substantially resemble the magnetic station shown in FIG. 4. The automated system may, for example, prepare samples with emulsions according to steps described above, and automatically place the sample tubes, without direct human interaction, containing the sample into magnetic stations to thereby separate analyte from other sample material for processing.

FIG. 6A-6D shows the separation of analyte from a portion of sample 605 inside a vessel 601. In particular, the figure shows hydrogel beads 607 bound with analyte being separated from a portion of sample 605 using a magnet 611. The sample comprising the analyte 605 and hydrogels 607 comprising magnetic particles are combined and near-instantly and

simultaneously separated into partitions 609 comprising a single hydrogel and a portion of the sample. The analyte is bound to the magnetic particles within each partition from the hydrogel. The presence of the magnet 611 near a side of the vessel 601 causes the magnetic particles to move towards the side of the vessel 601 nearest the magnet 611. As such, hydrogel beads 607 bound with analyte are aggregated inside the vessel 601. Once aggregated, the portion of the sample 605 not comprising the hydrogel beads can be easily and effectively removed from the vessel 601, for example using a pipette, or by dumping the contents of the vessel, without centrifugation.

What is claimed is:

1. A method comprising:

combining, in a vessel, hydrogel beads comprising magnetic nanoparticles and molecular binders with a sample comprising analyte;

partitioning the sample in the vessel using the hydrogel beads;

binding analyte to the molecular binders inside the partitions; and

separating bound analyte from a portion of the sample using a magnet.

2. The method of claim 1, wherein separating bound analyte is performed with the bound analyte inside the partitions.

3. The method of claim 2, wherein separating comprises segregating intact partitions from broken partitions and unwanted sample material.

4. The method of claim 1, wherein the separating step comprises contacting the magnet to an exterior surface of the vessel thereby causing bound analyte to associate with said surface.

5. The method of claim 4, further comprising pipetting the portion of the sample from the vessel while contacting the magnet with the surface of the vessel to thereby separate the portion of the sample from the bound analyte.

6. The method of claim 1, wherein the portion of the sample comprises crude sample extract.

7. The method of claim 1, wherein each one of the hydrogel beads comprises a plurality of magnetic nanoparticles.

8. The method of claim 7, wherein each one of the hydrogel beads comprises a hydrogel scaffold comprising polyacrylamide, bis-acrylamide, agarose, poly-ethylene-glycol, or polystyrene.

9. The method of claim 1, wherein the magnetic nanoparticles comprise a ferromagnetic element.

10. The method of claim 1, wherein the magnetic nanoparticles comprise metal oxides.

11. The method of claim 1, wherein the molecular binders comprise oligos, the oligos comprising a capture ligand for binding nucleic acid or protein.

12. The method of claim 11, wherein the oligos comprise a barcode and/or a unique molecular identifier.

13. The method of claim 1, wherein the molecular binders are attached to a hydrogel scaffold of the hydrogel beads via covalent bonds.

14. The method of claim 1, wherein the hydrogel beads comprise an agent that improves visibility of said beads within the vessel.

15. The method of claim 1, wherein partitioning the sample comprises vortexing the vessel.

16. The method of claim 1, wherein the hydrogel beads are at least 10 micrometers.

17. The method of claim 1, wherein the magnetic nanoparticles are approximately 10 nanometers in size.

18. A reagent comprising:

a plurality of hydrogel beads, each of the hydrogel beads comprising:

a hydrogel scaffold comprising magnetic nanoparticles embedded therein; and

molecular binders attached to an exterior surface of the hydrogel scaffold, the molecular binders comprising capture ligands for binding nucleic acid or protein.

19. The reagent of claim 18, wherein the molecular binders comprise a barcode and/or a unique molecular identifier.

20. The reagent of claim 18, wherein the magnetic nanoparticles are ferromagnetic.

21. The reagent of claim 18, wherein the hydrogel beads are at least 10 micrometers and the magnetic nanoparticles are less than 10 nanometers.

22. A method comprising:

combining hydrogel beads and magnetic particles in a mixture, thereby associating the magnetic particles with a plurality of the hydrogel beads;

combining, in a vessel, the plurality of hydrogel beads and a sample comprising an analyte;

binding the analyte to the magnetic particles inside the partitions; and

separating bound analyte from the sample using a magnet.

23. The method of claim 22, wherein the magnetic particles are less than 5 micrometers.

24. The method of claim 22, wherein the magnetic particles are silica coated.

25. The method of claim 22, wherein the magnetic particles are embedded in the hydrogel beads.

26. The method of claim 22, wherein the hydrogel beads comprise pores and wherein the combining step comprises disposing magnetic particles within the pores of the hydrogel beads.

27. The method of claim 22, wherein the combining step comprises associating the hydrogel beads and magnetic particles by charge interactions between the hydrogel beads and magnetic particles.

28. The method of claim 27, wherein the combining step comprises associating the hydrogel beads and magnetic particles using oligonucleotides disposed on the surface of the hydrogel particles.

29. The method of claim 22, wherein in the combining step the vessel is a microcentrifuge tube.

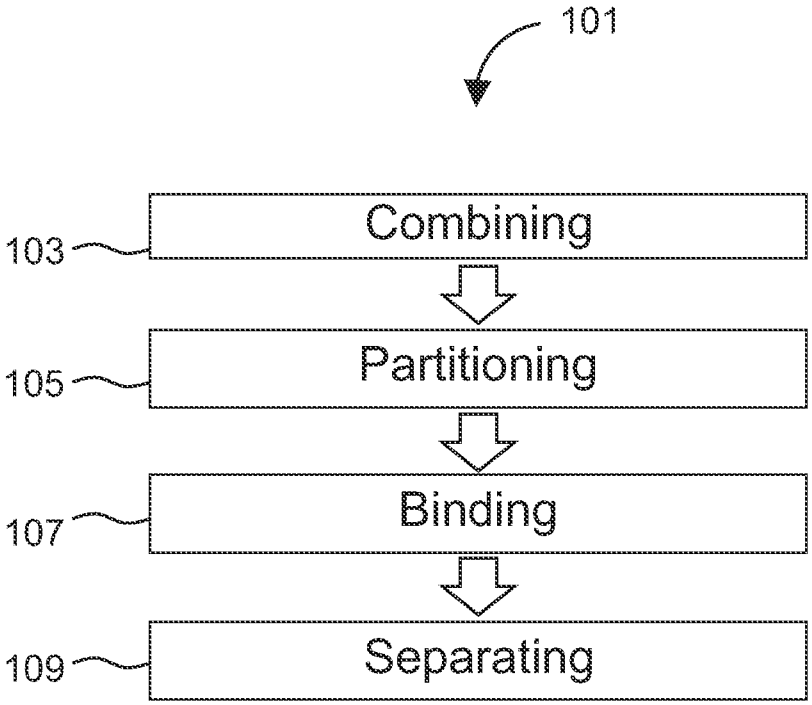
101
Combining
103
Partitioning
105
Binding
107
Separating
109

FIG. 1

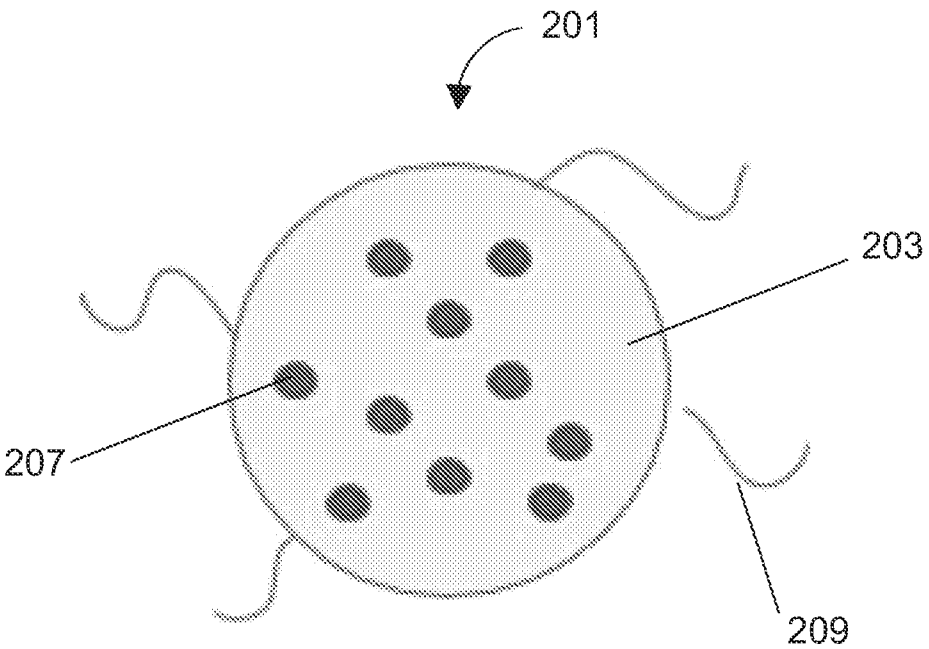
201
203
207
209

FIG. 2

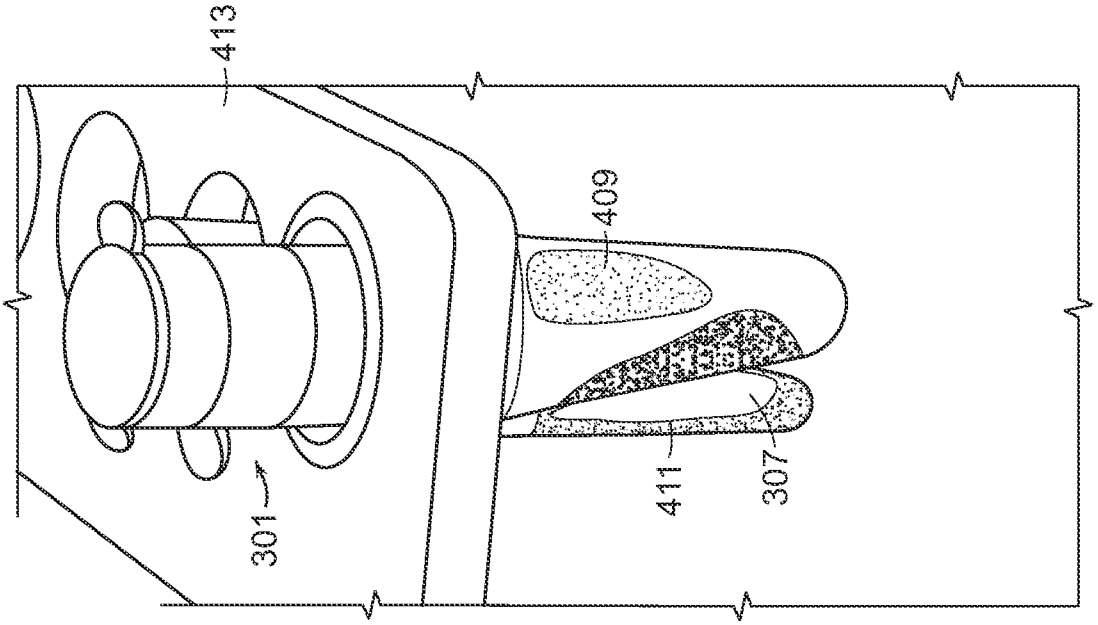

FIG. 4

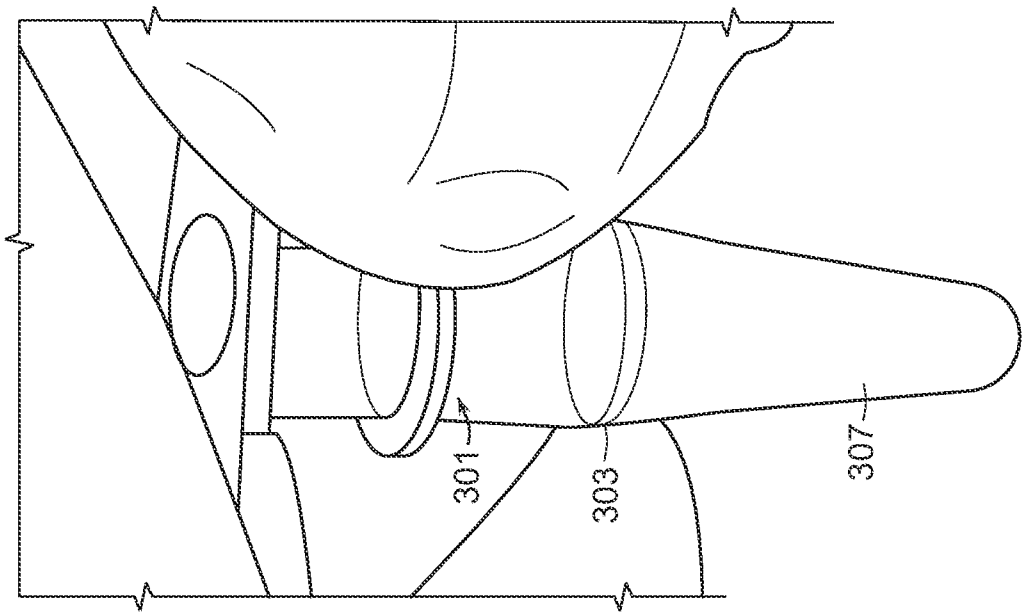

FIG. 3

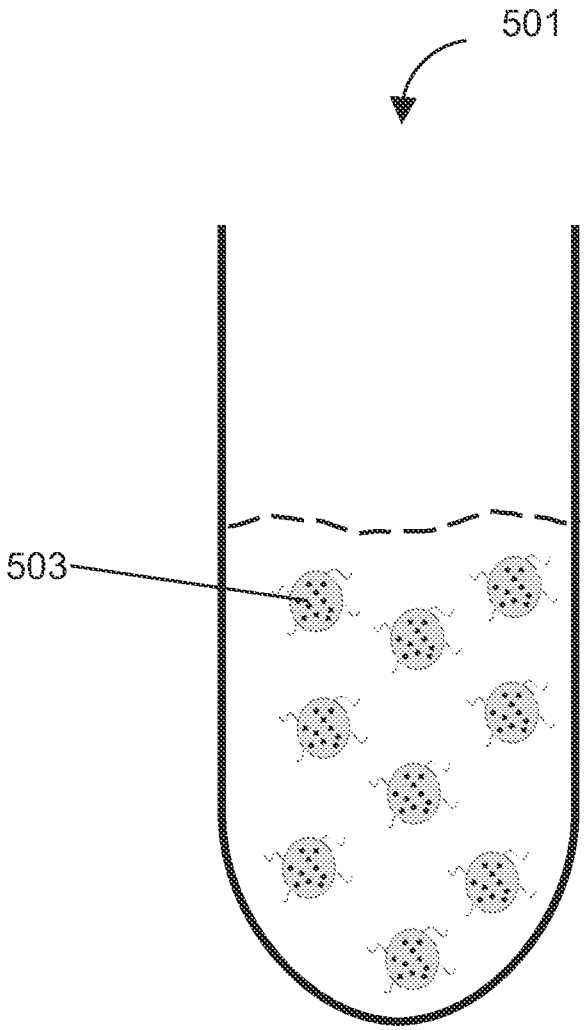

FIG. 5

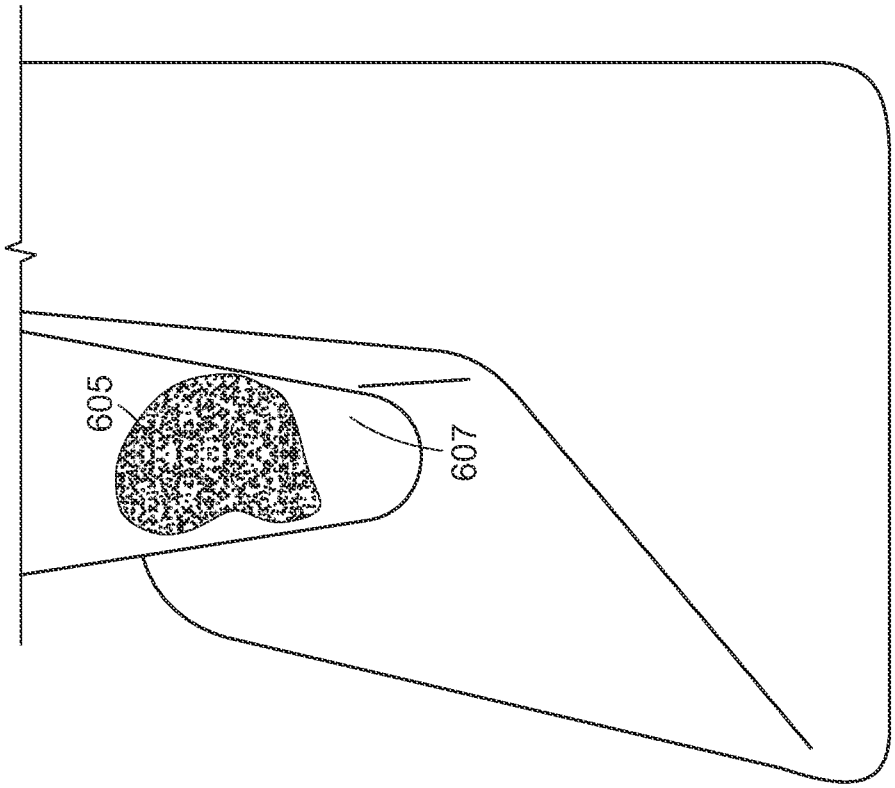

FIG. 6B

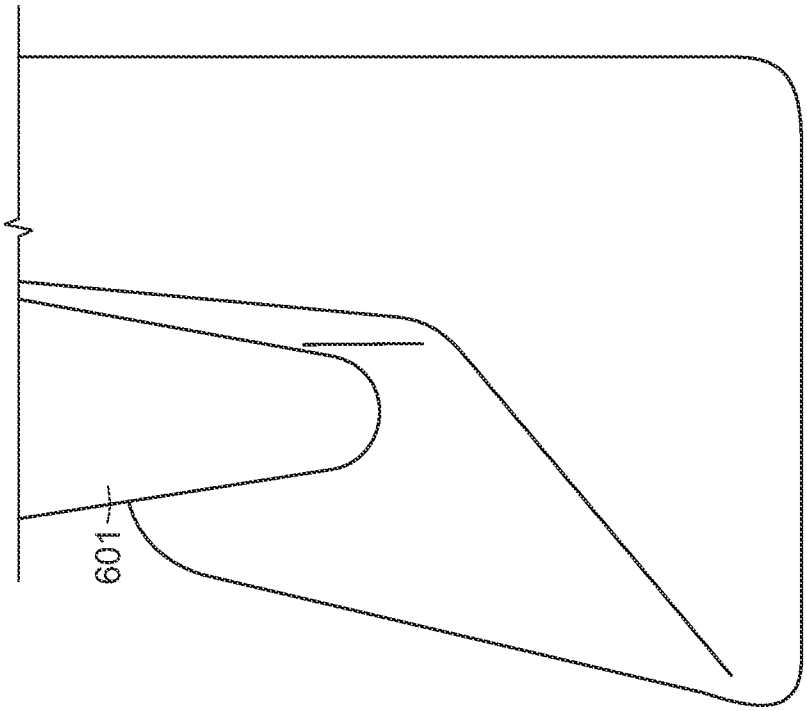

FIG. 6A

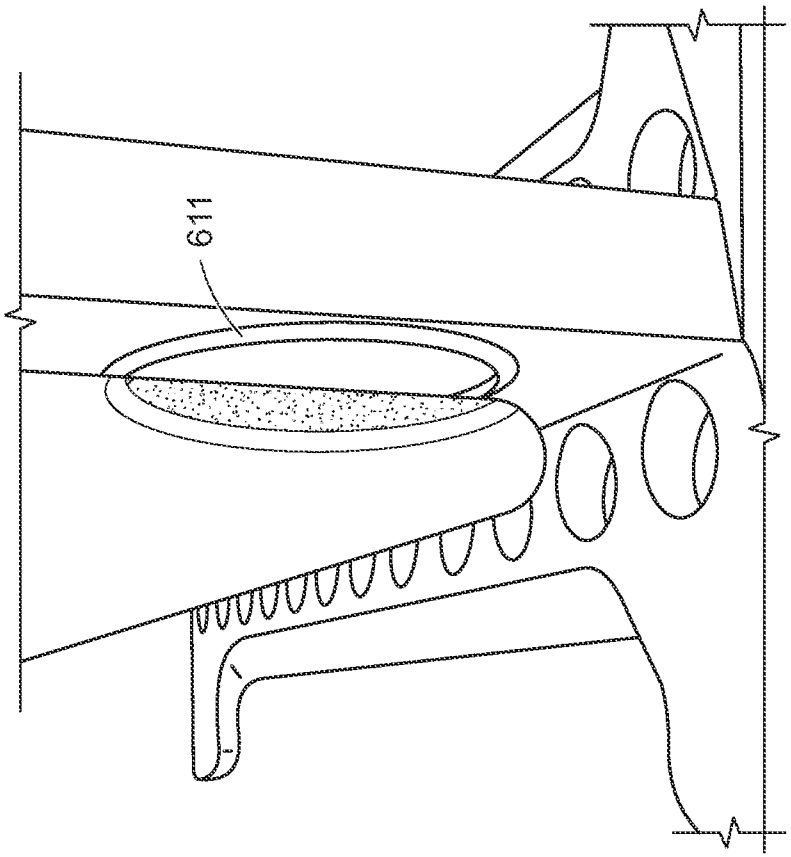

FIG. 6D

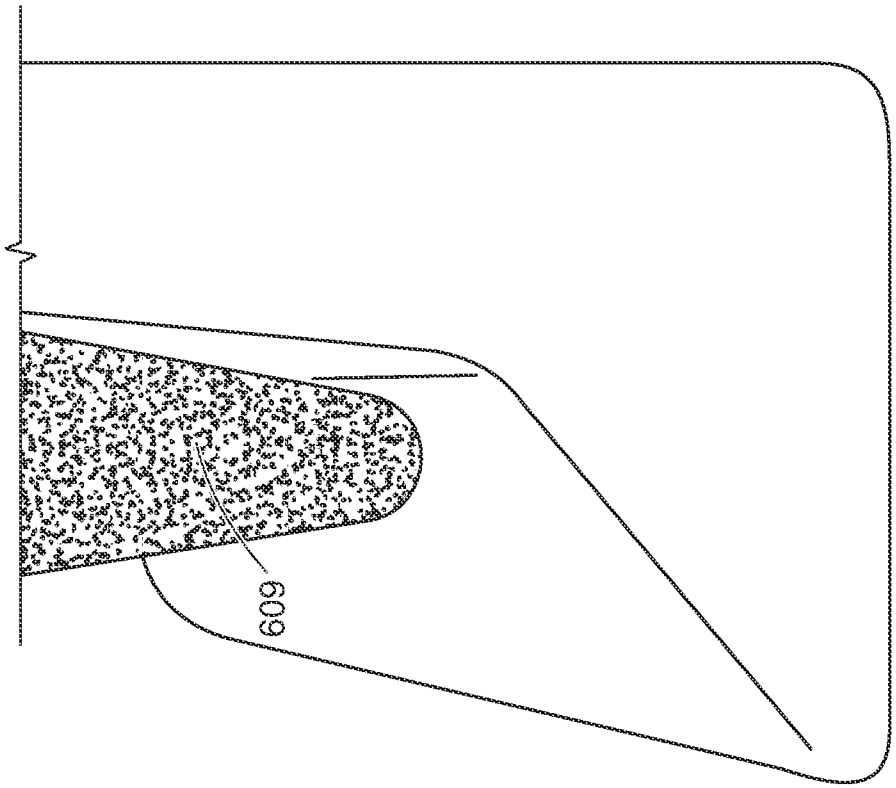

FIG. 6C

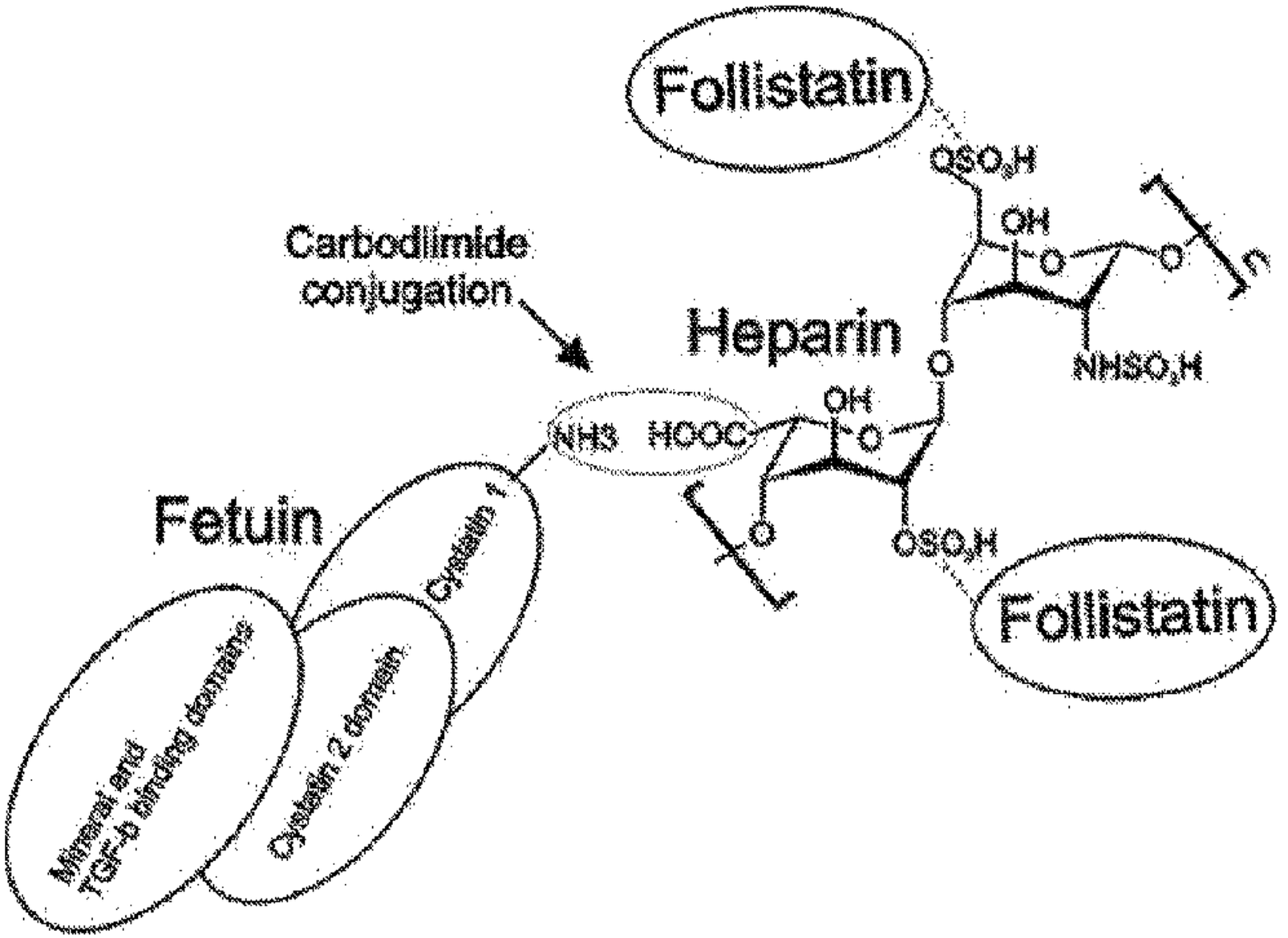
Follistatin
OSO3H
OH
O
O
Carbodiimide
conjugation
Heparin
NHSO3H
OH
NH3
HOOC
O
Fetuin
Cystatin 1
O
OSO3H
Follistatin
Mimecan and
TGF-b binding domains
Cystatin 2 domain

FIG. 1