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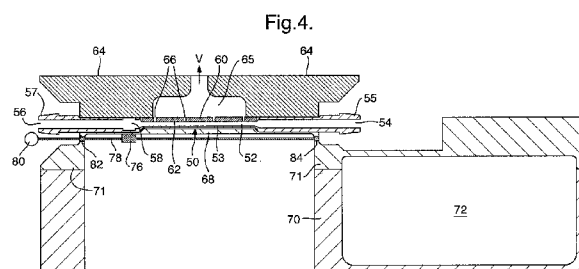
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(57) Abstract: A method of processing of a sample mixed with paramagnetic particles comprises exposing the sample mixed with paramagnetic particles to a magnetic field to draw the paramagnetic particles, and then forming an ultrasound standing wave to drive the paramagnetic particles into one or more pressure nodes. Apparatus is also described for the analysis of biological samples which comprises a chamber, an acoustic transducer capable of creating at least one pressure node in a fluid contained in the chamber, and one or more magnets associated with the chamber to attract paramagnetic materials contained in a sample.

Description

ULTRASOUND & MAGNETIC METHOD

- [0001] This invention relates to methods and apparatus particularly for extraction and separation of target material. Although not limited to diagnostic reactions or cell separation applications it is particularly suitable for these applications.
- [0002] Diagnostic assays as well as cell separation techniques often utilise a ligand to isolate a specific analyte or cell through a specific marker on the cell surface. The ligand is attached to a solid phase to enable the specific cell or analyte to be retained, whilst non-specific items are removed by washing them away.
- [0003] Antibodies are one widely used example of a ligand, but others include nucleic acid probes. In some cases charge on a surface is sufficient as used in capture of released DNA on a silica particle.
- [0004] A variety of solid phases can be employed, but particles are a popular and widely utilised format. Particles with a polystyrene outer shell are particularly favoured because their density is close to water and this allows easy mixing and because ligands and other biological capture molecules easily bind to the surface.
- [0005] In each case it is necessary to remove the non-bound materials in the sample from those bound to the solid phase. Centrifugation or filtration methods can be used with polystyrene particles but increasingly use of a magnetic field on a particle with a metal oxide core is preferred. Such particles are designed with a paramagnetic core of ferrite material, with a coat of polystyrene surrounding it to produce the preferred size and provide a surface for the ligands to bind.
- [0006] Paramagnetic cores are chosen because they have no magnetic attraction of their own and therefore particles can remain monodispersed in solution, but if a magnetic field is applied, they are attracted and drawn to the magnet. Typically the reactions are performed in a tube or micro titre well. Particles are added to the sample in the tube without any magnetic field present and allowed to mix, binding any analytes or cell surface antigens in the sample. A magnetic field is applied usually by bringing a solid state magnet to the side or bottom of the tube. The particles are drawn to the magnet and the supernatant can be siphoned off, usually by pipette. The magnetic field can then be removed and washing buffers added. The process is usually repeated 3-5 times to ensure efficient washing of the particles, before the next stage of the procedure is followed.
- [0007] Whilst the method is simple and magnets are widely available, it does have a number of limitations.
- [0008] Firstly, the particle size range is very limited. As previously mentioned, polystyrene particles are favoured in diagnostic applications due to their density close to water, i.e.

1. At this density particles can be easily be kept suspended in a sample as they sediment very slowly. It also ensures that they are suspended equally throughout the volume of the liquid sample, maximising the likelihood of the ligand meeting a target to bind. In practice the rate of interaction is determined by Brownian motion as the targets are small and move through the liquid to meet the particles / ligands. The rate of interaction determines the incubation period and therefore the time for the assay or protocol. In general all methods seek to speed up the protocol time to increase throughput and minimise hands on time.

[0009] The density of the polystyrene particles are not affected by size and size ranges of polystyrene particles range from 10 nm to 100µm in diameter with benefits in different applications. For example, in-vivo imaging methods prefer particles of 10-50 nm, which will be actively taken into cells by methods such as phagocytosis, latex agglutination methods prefer particles of 100 nm due to the even, milky background which aids visualising speckling in a positive reaction, membrane lateral flow applications prefer coloured particles in the range of 100-200 nm due to their ability to be carried through pores in a porous material such as nitrocellulose by capillary action and other methods prefer 10-100 µm diameter particles due to their ease of separation using a filter membrane.

[0010] Paramagnetic particles need to achieve a combination of slow sedimentation rate to enable capture of target, with rapid attraction by a magnetic field to allow separation. The metal oxides used for the paramagnetic core are dense and tend to sediment rapidly. Consequently paramagnetic particles in diagnostic or cell separation applications have a thick coating of polystyrene to counteract the density and retain particle buoyancy. Ferrite is the most commonly used material for a paramagnetic core and most commercially available particles are available in a narrow size range of 1.0 to 5 µm diameter. Commercial cell separation products such as the Dynal™ range from Invitrogen or the Captivate™ range from LAB M have standardised at 2.7 µm diameter for bacterial capture and 4.5 µm for larger cells. If the particles are smaller than 1.0 µm in diameter, the paramagnetic core is too small to allow a magnetic field to rapidly draw them to the wall of the tube, thus increasing the assay time. If they are larger than 5 µm, they sediment rapidly and do not enable efficient capture of target in the bulk of the sample. Consequently paramagnetic particles have only been applied to a limited number of applications compared to non-magnetic polystyrene particles.

[0011] In addition problems can arise when particles coated with biological materials are pushed or pulled in too close a proximity to another surface. Design of a particle for use in biological applications must seek to balance the various charges in play. The native particles tend to be hydrophobic and in their natural state, in an aqueous environment they will clump together with other hydrophobic materials. Part of the aims

of particle design is to neutralise these forces by coating with non-reactive proteins such as Bovine Serum Albumin or materials with complex protein mixes, such as milk powder. Proteins also have regions of positive and negative charge, but in general the charges are balanced in a coating regime. If however they encounter another surface or particle, a localised charged region can cause sticking. Proteins are also particularly prone to stick to surfaces such as plastics or glass which also have hydrophobic surfaces. If a paramagnetic particle coated in biological materials is drawn to the surface of a plastic tube, vigorous methods are required to re-suspend them after the magnetic field is removed. These include pipetting, vortexing and shaking. In some cases the particles do not return to their mono-dispersed state, thereby removing ligand – antigen complexes on their surface from ensuing reactions. Other particles do not leave the wall of the tube or are lost in the aspiration processes. Particle recovery levels are not widely reported, but there are clearly significant losses. Studies have reported losses of 50% of total captured *Listeria* from ham samples using an immunomagnetic separation method (Hudson et al, J Appl Microbio, 90:614-621). The inventors are not aware of any definitive study reporting recovery or even explaining the mechanism for particle losses, but discussions with researchers in the field confirm that losses of 20-80% have been observed with different sample types.

- [0012] As mentioned above vigorous mixing is required to re-suspend paramagnetic particles after application of a magnetic field. Antibody coated magnetic particles are widely used to capture pathogenic bacteria or in DNA purification as a step in analysis using the Polymerase Chain Reaction (PCR) or other nucleic acid amplification method. Vigorous mixing can lead to aerosol generation, with associated health risk in the case of pathogenic bacteria and a cross contamination risk in PCR analysis.
- [0013] Complex instrumentation is required to automate a process designed around paramagnetic particles. Automation is seen as an essential requirement for wide scale adoption, as manual methods are cumbersome, time consuming and limited to only a small number of samples per day. Current instrumentation approaches use solid state magnets which are mechanically moved to bring a field into contact or away from the tube containing the particles. This is combined with robotic pipetting stations that aspirate and add liquids to tubes to re-suspend the particles. Due to the density of the particles constant mixing and agitation is required to prevent them sedimenting in the stock reservoirs. Automated instruments for magnetic particles are thus few in number, compared to other automated platforms and are relatively expensive.
- [0014] So far a simple instrument that overcomes the problems of cost, complexity, non-specific sticking, effective washing and avoidance of aerosols has not been available.
- [0015] Other methods of manipulating particles have been investigated. An ultrasound or acoustic standing wave field is capable of localising particles within a liquid at either

the pressure nodes or antinodes of the field. Localisation is dependent upon a number of different factors including the relative densities and compressibility of the particles and the fluid.

- [0016] An acoustic standing wave field is produced by the superimposition of two waves of the same frequency travelling in opposite directions either generated from two different sources, or from one source reflected from a solid boundary. Such fields are characterized by regions of zero local pressure (acoustic pressure nodes) with spatial periodicity of half a wavelength, between which areas of maximum pressure (acoustic pressure antinodes) occur.
- [0017] Ultrasound is sound with a frequency over 20,000 Hz. It has long been established that acoustic radiation force generated in an ultrasound standing wave resonator can bring evenly distributed particles/cells in aqueous suspension to the local pressure node or antinode planes. The radiation force arises because any discontinuity in the propagating phase, for example a particle, cell, droplet or bubble, acquires a position-dependent acoustic potential energy by virtue of being in the sound field. Suspended particles tend therefore to move towards and concentrate at positions of minimum acoustic potential energy. The lateral components of the radiation force, which are about two orders of magnitude smaller than the axial, act within the planes and concentrate cells/particles in a monolayer. This phenomenon has successfully been used to aggregate clumps of particles or cells and to use flow of liquids to control the sequence of reactions and discriminate between positive and negative results in antibody labelling or cross linking (agglutination) reactions as described in
Patent Citation 0001: WO WO 2008/037993 A (PROKYMA TECHNOLOGIES LIMITED). 2008-04-03.
and
Patent Citation 0002: WO WO 2009/118551 A (PROKYMA TECHNOLOGIES LIMITED). 2009-10-01.
- [0018] According to the present invention a method of processing of a sample mixed with paramagnetic particles includes forming an ultrasound standing wave to drive the paramagnetic particles into one or more pressure nodes and exposing the sample mixed with paramagnetic particles to a pulling force to draw the paramagnetic particles.
- [0019] In various embodiments of the invention and at various stages of the method forming an ultrasound standing wave and exposing the paramagnetic particles to a pulling force may be carried out together or in sequence, in either order, as described below.
- [0020] In one embodiment of the invention a method of processing a sample comprises the steps of
- mixing the sample with paramagnetic particles with surfaces that capture

materials of interest;

- introducing at least part of the sample material in a fluid into a chamber, establishing at least one pressure node in the chamber by means of an acoustic standing wave in the fluid;
- aggregating the sample at the node, reducing the strength of or eliminating the acoustic standing wave and subjecting the paramagnetic particles to a pulling force to draw them towards a wall of the chamber; and
- re-establishing the acoustic standing wave to drive the paramagnetic particles back into pressure nodes.

[0021] The pulling force pulls the paramagnetic particles towards a wall of the chamber when the strength of the acoustic standing wave is reduced or the acoustic standing wave eliminated. Ideally the paramagnetic particles are washed while they are held by the pulling force at a wall of the chamber, preferably by passing a laminar fluid flow over the paramagnetic particles. By drawing the paramagnetic particles towards a wall of the chamber and holding them close to a wall of the chamber, a laminar flow of fluid will tend to wash unbound compounds from the chamber, but the particles themselves are not flushed out of the chamber because the weak force of the laminar flow close to a wall is insufficient to overcome the magnetic forces attracting the paramagnetic particles to the wall.

[0022] In one embodiment the standing acoustic wave is reduced or eliminated and then re-established on a plurality of occasions before the particles are flushed from the chamber.

[0023] Although the pulling force can be gravitational it is preferred that it is exerted by a magnetic field. Applying a magnetic field to the chamber as the sample is admitted to the chamber dampens kinetic energy of the paramagnetic particles and speeds up aggregate formation.

[0024] A preferred alternative to cycling, reducing or eliminating the standing acoustic wave and then re-establishing it is either to increase or decrease the magnetic field on a cyclic basis while paramagnetic particles are held in an acoustic wave of constant strength.

[0025] Ideally a magnetic field is applied continuously to the chamber while any paramagnetic materials are present in the chamber. This has the advantage that it acts as a brake on the paramagnetic particles when they enter the chamber, reducing the propensity of the particles to be flushed from the chamber with any fluid flow.

[0026] By following the measures of the previous two paragraphs aggregates of greater mass than single particles are formed and this in turn enhances the effect of a magnetic field.

[0027] Other measures that can be taken include:

- washing the paramagnetic particles while held at in an acoustic standing

wave; and

- gathering the paramagnetic particles in a magnetic field after the particles have passed through a chamber in which pressure nodes may be formed.

[0028] The method according to the invention is particularly appropriate for use with a sample containing biological particles, particularly bacteria. Thus the paramagnetic particles should have surfaces that will capture biological particles.

[0029] A method can be part of a process to extract biological cells from samples containing materials that can act as inhibitors for detection methods and is especially useful, for example, when the sample is one to be tested for *Mycobacterium tuberculosis*.

[0030] A particular aspect of this invention is the possible use in the method of a disposable chamber and disposing of the chamber after processing each sample.

[0031] In another aspect of the invention separation apparatus for use in the analysis of biological samples comprises at least a first chamber, an inlet and an outlet to said chamber, at least one acoustic transducer capable of creating at least one pressure node in a fluid contained in the chamber and mounted against a wall of said chamber, and one or more magnets associated with the apparatus to attract paramagnetic materials contained in a sample. The magnets may be permanent magnets, electromagnets or solid state magnets, or any other device generating a magnetic field.

[0032] An ultrasound standing wave is highly efficient in pushing the paramagnetic particles off the chamber surfaces and back into the nodal plane or planes. At which point, reactions can be controlled, by introducing buffers containing reagents, which actively mix and react. Positioning of transducers to generate ultrasound standing waves in chambers of different geometries have been widely described and include tubular or capillary shapes, circular, square, rectangular or hexagonal to mention a few. This description shall refer to chambers with flat walls on the base and ceiling of the chamber, but the invention is not restricted to these presentations.

[0033] The one or more magnets are normally positioned on the outside of a wall of said first chamber which is normally the acoustic reflecting wall of the chamber.

[0034] A close contact (coupling) is required between the transducer and the chamber to allow transfer of acoustic energy from the transducer into the chamber. Multilayer chambers have been described where the coupling is achieved using a physical contact mechanism, such as an adhesive or a liquid coupling layer, such as glycerol or oil. In a particularly interesting aspect of the invention the wall opposite the acoustic reflecting wall comprises a flexible thin film material. In such a case the acoustic transducer can be affixed to the outside surface (facing into the chamber) of the flexible thin film material. Typically the thin film material used is about 20µm thick. Using such a thin film material the acoustic transducer can be coupled to the chamber without a physical contact mechanism such as an adhesive or liquid coupling layer, such as glycerol or

oil. Furthermore the apparatus is operable over a range of frequencies to generate aggregates; changes in frequency are observed to assist in the formation of aggregates.

[0035] One way of achieving the coupling of the transducer to the thin film material without an adhesive or liquid coupling layer is to reduce the pressure on the outside surface of said thin film material. Another mechanism is to use an elastic film and to press the layer across the transducer to stretch the film and form a tight fit.

[0036] After passing through the chamber the paramagnetic particles can be gathered in the outlet by placing a magnet against the outlet to collect them. For this purpose a small second chamber can be provided in the outlet with a magnet positioned against its wall. Ideally the chamber and the inlet and outlet are disposable and are mountable in a frame.

[0037] Thus in a third aspect of the invention a chamber for use in the analysis of biological samples is characterised in that said chamber is a one piece moulding with inlet and outlets, side walls and an acoustic reflecting wall, and a thin film wall disposed opposite said acoustic reflecting wall and attached to the side wall by lamination. Ideally said moulding consists of materials which can be produced at low cost by techniques such as injection moulding such as polystyrene, polycarbonate, acrylic or other polymeric plastic materials.

[0038] Best results are obtained if the thin film material is about 20µm thick.

[0039] Use of thin film enables an acoustic transducer to be attached to the outside surface of the thin film without adhesive.

[0040] Using a thin film enables the chamber to be operated over a range of frequencies to form the aggregate.

[0041] Other features of such a chamber may include:

- A magnet placed against the outlet to collect paramagnetic particles in its associated magnetic field after the paramagnetic particles have passed through the chamber;
- The outlet having a second chamber with a magnet placed against the outlet is positioned against the wall of said chamber;
- The acoustic transducer attached to the outside surface of the thin film without adhesive applying a reduced pressure to the outside surface of the thin wall, by using a liquid coupling layer, reduced pressure or by plastic deformation of the thin wall;
- One or more magnets mounted adjacent to but outside the reflecting wall;
- The chamber mounted in a frame;
- An air gap provided between the reflecting wall of the chamber and a frame member;
- One or more magnets above the chamber with the thin film being the lower

wall; and

- The one or more magnets applying magnetic fields to different parts of the chamber at different times.

[0042] A key aspect of the invention is the use of ultrasound in a chamber to resuspend paramagnetic particles that have already been captured onto a surface by one or more magnets ; the resuspension is near instantaneous and highly efficient and the process can be repeated as often as required to facilitate washing and reagent addition/removal to/from the particles. The ultrasound resuspension overcomes one of the key disadvantages of magnetic separation, namely clumping of the particles in a strong magnetic field and subsequent poor resuspension. This is a highly efficient way of resuspending paramagnetic particles without introducing vigorous mechanical means such as agitation/shaking/repeated pipetting. The combination of magnetic capture and ultrasound resuspension in a sealed chamber is an important advance in diagnostic technology as it allows processing of the paramagnetic particles with no operator intervention or use of mechanical devices. Furthermore it has been found that by using the method of this invention, larger ultrasound chambers can be used than in the known processing systems for capture and holding of particles with minimal particle loss, leading to substantially higher throughput. This outcome is surprising.

[0043] Ultrasound resuspension has also been shown to be useful in the absence of a magnetic field when the particles have settled in the chamber under gravity alone. Thus the combination of particle settling under gravity with or without an additional magnetic field present and subsequent ultrasound resuspension is an additional aspect of the invention.

[0044] The invention has the further advantage over previous approaches for processing of paramagnetic particles to which biological moieties are attached and that is in the case of, for example, bacterial pathogens, the method provides significant operator safety as once a sample is introduced into the enclosed chamber, subsequent procedures can take place in the chamber minimizing the possibility of operator exposure to the sample.

[0045] The materials of interest may be biological moieties including proteins, enzymes, hormones, bacterial and eukaryotic cells; and in that case the paramagnetic particles have surfaces that have been modified to capture specific biological materials. However the technique can also be applied, for example, to a sample containing DNA and possibly to the extraction of materials generated in chemical reactions.

[0046] The method of this invention may be used as part of a process of separation of biological cells from assay inhibitors. In one application the sample is sputum which may contain *Mycobacterium tuberculosis*. In another application the sample is a blood sample.

[0047] At each stage the paramagnetic particles may be washed while held at a pressure

node.

[0048] In another aspect of the invention apparatus for the analysis of biological samples comprises at least one chamber, an acoustic transducer capable of creating at least one pressure node in a fluid contained in the chamber, and one or more magnets associated with the chamber to attract paramagnetic materials contained in a sample.

[0049] An ultrasound standing wave is highly efficient in pushing the paramagnetic particles off the top or bottom chamber surfaces (where the transducer is attached to the outside of the bottom surface and the top surface is the reflector) and back into the nodal plane or planes. At which point, reactions can be controlled, by introducing buffers containing reagents, which actively mix and react.

[0050] The invention provides a simple way to improve the efficiency of liquid mixing with the paramagnetic particles by turning off the ultrasound standing wave and allowing the particles to pull towards a wall, with resultant deformation of the aggregate structure and then to reapply the standing wave to reform an aggregate that is held against a flow. This process can be cycled multiple times ensuring unimpeded access of liquids to the centre of the aggregate and more efficient mixing.

[0051] Other advantages include the smaller size of paramagnetic particles that can be employed. Ultrasound forces affect particles on the basis of their size and density. When using ultrasound forces to manipulate polystyrene particles, sizes of 5 μm or larger are preferred. When using paramagnetic particles, the increased density allows improved performance on 1.0 μm particles. This leads to the ability to use larger particle numbers and consequently a larger surface area to volume ratio, with improvements in assay performance, speed and sensitivity. Also, due to the ultrasound force lifting paramagnetic particles that would otherwise sediment; larger paramagnetic particles can be employed if required.

[0052] Unexpectedly, the force of the ultrasound pushing paramagnetic particles into a nodal plane can exceed the strength of a magnetic field pulling paramagnetic particles to the floor or ceiling of the chamber. This has allowed the reaction to be controlled by cycling the ultrasound power on or off whilst leaving the magnetic field active. This has an advantage of minimising particle loss when a flow is applied as any paramagnetic particles escaping the ultrasound field are attracted to the floor or ceiling of the chamber. This has also led to the observation that the capture efficiency in the ultrasound node is higher if both the ultrasound standing wave and the magnetic field are applied at the same time. The capture efficiency is defined as the ability of the aggregate of paramagnetic particles to withstand flow rates without loss of paramagnetic particles. Cycling of either the strength of the ultrasound standing wave or the magnetic field on or off aids in mixing and washing of the paramagnetic particles by moving them through the liquid in the chamber. The strength of the magnetic field

can be controlled electronically if using an electromagnet, or by varying the proximity of a solid state magnet to the chamber wall.

[0053] The combination of forces has also allowed the inventors to use a larger chamber than that used in known ultrasound devices and thus increase the volume of samples processed in a short time frame as required for diagnostic tests. Previous attempts to fabricate an ultrasound chamber that could hold 1 ml of sample were unsuccessful and inefficient, as this required a large ultrasound transducer that consumed power and heated the chamber to a temperature that denatures biological materials. In larger ultrasound chambers, the ability to hold non-paramagnetic particles against a flow was also observed to be poor and the recoveries of the target biological materials were less than 50%. Using a combination of magnetic fields with ultrasound standing waves according to the invention has allowed the fabrication of a chamber to process 1 to 10 ml of sample volume, with greater particle holding power and using less power.

[0054] Reactants may be introduced into the chamber with the sample or separately. As used herein, analyte or biological moiety is particularly intended to mean a bacterial cell, blood cell, blood platelet, cell fragment, spore, plasmid or virus, but also includes synthetic particles which may or may not be modified, or coated, with one or more different chemical or biological moieties or synthetic derivatives thereof. Examples of synthetic particles include, but are not limited to, polymers, such as latex and polystyrene, composites such as gold coated polystyrene, particles with a paramagnetic core and glass/silica beads that may or may not be coated with proteins, capture moieties, recognition elements, ligands, amplification moieties or other chemical or biological agents.

[0055] It should be emphasised that where flow rates and voltages are given in this specification, they relate to flow rates and voltages used by the inventors on their apparatus. The appropriate flow rates and voltages may vary depending on the exact configuration and parameters of the ultrasound system in use, and appropriate calibration will be required. This step is within normal experimental skills.

[0056] The invention will now be described, by way of example, with reference to the accompanying drawings, in which:

[0057] Figure 1 shows a side section and Figure 2 a top view of a separation apparatus according to the invention, which is similar to the ultrasound device described in L. A. Kuznetsova et al, Langmuir 2007, 23, 3009 – 3016, but modified in accordance with this invention;

[0058] Figure 3 is a longitudinal section through a separation apparatus with a disposable chamber on the line B-B' of figure 4; and

[0059] Figure 4 shows a transverse section through the separation apparatus of Figure 3.

[0060] In Figures 1 and 2 the separation apparatus comprises a circular stainless steel

support 11, with an internal circular lip 11a on which is mounted, from below a thin stainless steel layer 15. The internal circumference of the lip and the layer 15 define the side and bottom of a chamber 14. An inlet 16 and an outlet 17 are formed through the support 11 and lip 11a to the chamber 14. A piezoelectric ultrasound transducer 12 is mounted below the layer 15 such that the centre of the transducer lies on the same axis as the centre of the chamber 14. A glass or quartz glass reflector 13 is mounted above the chamber 14; the diameter of the reflector is greater than that of the chamber 14 so that it can be sealed to the lip 11a, and seal off the chamber. The arrangement is such that the layer 15 couples the transducer 12 to the chamber 14 and the reflector 13 will allow for the creation of standing waves in any liquid in the chamber 14, when the ultrasound transducer is turned on.

[0061] The gap between the layer 15 and reflector 13 is a multiple of one half the wavelength of the intended mean frequency of the input to the ultra sound transducer. In this particular example the transducer 12 has a nominal frequency of 1.5MHz, and a gap of 500µm across the chamber 14 between the layer 15 and the reflector 13 represents one ½ wavelength.

[0062] In use the chamber 14 would be filled through the inlet 16 by pumping from a peristaltic pump 19 a sample to be mixed in a fluid comprising a standard buffer solution. In this example, the sample is blood suspected of containing bacteria. Once the chamber 14 is filled the pumping is stopped, the ultrasound transducer 12 can be turned on forming one or more pressure nodes in the fluid. Below the chamber is disposed a magnet 20 (which may be a permanent magnet, an electromagnet or a solid state magnet).

[0063] Example 1 describes the evaluation of the chamber with paramagnetic particles; Example 2 describes the isolation of TB (*Mycobacterium tuberculosis*) from a 3 ml treated sputum sample and the use of the separation apparatus of Figures 1 and 2 to isolate the target TB bacteria.

[0064] **Example 1:**

[0065] Step 1. 12.5µl of Lodestar Str-A beads (2.7 µm paramagnetic particles, obtainable from Polymer Laboratories, UK) were added to 87.5µl of 0.01M PBS pH7.4;

[0066] Step 2. A permanent magnet 20 was placed below the chamber 14 underneath the transducer 12;

[0067] Step 3. The ultrasound was switched on at a frequency of 1.432 MHz;

[0068] Step 4. The sample mixed with the Lodestar beads was loaded into the ultrasound chamber at a flow rate setting of 0.75 on a Gilson Miniplus 3 peristaltic pump 19;

[0069] Step 5. An aggregate formed as the particles flowed into the chamber and they were continuously captured into the aggregate for 100 seconds;

[0070] Step 6. The liquid inlet was switched from the sample to a wash buffer consisting of

0.01M PBS pH7.4 at the entry tube 16;

[0071] Step 7. The PBS was flowed through the chamber 14 for 40 seconds at a setting of 0.75 on the Gilson Miniplus 3 peristaltic pump 19;

[0072] Step 8. The ultrasound standing wave was deactivated by switching off power to the transducer 12 and the Lodestar paramagnetic particles were drawn to the floor of the chamber by the magnet in less than 1 second. The morphology of the aggregate changed as the particles compacted and moved according to the magnetic field;

[0073] Step 9. Reactivating the ultrasound standing wave by resupplying power to the transducer 12 resulted in the particles rising off the floor of the chamber 14 and forming a typical aggregate 18 that had a circular appearance when viewed from above;

[0074] Step 10. Switching the power to the signal generator on and off allowed a repeated controlled sedimentation and levitation of the particles between the compacted and elevated states.

[0075] **Example 2**

[0076] Isolation and visualisation of target TB Bacteria.

[0077] Step 1. Using a commercial kit (TB-Beads) available from Microsens Medtech Limited, London UK, a sputum sample taken from an infected patient was first thinned and decontaminated according to the manufacturer's instructions in the kit;

[0078] Step 2. Paramagnetic beads functionalized with ligands that bind TB (*Mycobacterium tuberculosis*) as supplied in the commercial kit were mixed with 3 ml of the thinned sputum sample;

[0079] Step 3. The ultrasound chamber 14, capable of holding 70 μ L of liquid was pre-filled with buffer. The ultrasound transducer was activated at a frequency of 1.49 MHz. A magnet 20 was positioned under the floor of the chamber 14;

[0080] Step 4. 3 ml of the thinned sputum sample/TB bead complex was introduced in the chamber 14 through inlet 16 in a continuous flow, and the particles along with any bound mycobacteria were observed to form into an aggregate. No particles were observed escaping at the other end through outlet 17. In this design of circular chamber 14 the aggregates 18 of particles were observed to form predominantly in one central aggregate;

[0081] Step 5. After 2 minutes, whilst the TB bead aggregates 18 were still in the chamber, the flow was stopped and the power of the ultrasound standing wave reduced by lowering the voltage on the transducer 12, allowing the particles to be drawn by the magnetic field created by the magnet 20 to the floor of the chamber;

[0082] Step 6. The chamber 14 was washed with distilled water using the peristaltic pump 19 at flow rate setting 8 with 3 ml of wash solution in 1 minute. The flow was stopped, the voltage across the transducer 12 increased to increase the power of the ultrasound

standing wave and the beads were observed to return to a central aggregate in the chamber 18;

[0083] Step 7. The captured mycobacteria on the magnetic beads were then stained by keeping the beads suspended in the chamber using the ultrasound standing wave and then flowing 1 ml of auramine stain into the chamber using the peristaltic pump 19 at flow rate setting 8, followed by 1 ml of potassium permanganate counterstain and finally 1 ml of distilled water wash. Finally the electromagnet and the ultrasound were switched off and the beads were removed from the chamber by pumping into an Eppendorf tube. Using a Pasteur pipette the beads were smeared onto the surface of a standard glass microscope slide, fixed in place on a heating block at 60°C for 10 minutes and then examined in a fluorescence microscope (LW Scientific, with blue LED excitation adaptor). The characteristic yellow rods of the bacilli were observed bound to the surface of the dark brown magnetic beads.

[0084] The process steps in Examples 1 and 2 can be summarised as

- using magnetism as an additional brake on paramagnetic beads flowing into the chamber to slow them so that the ultrasound forms aggregates more efficiently;
- once the aggregates had formed reducing the ultrasound and enhancing the magnetic pull due to the larger mass of paramagnetic beads in a clump;
- using magnetism to hold the beads in the dead volume of laminar flow to wash unattached compounds away (overcoming the lack of size specificity of ultrasound); and
- using ultrasound to push beads off the walls, whilst magnetic force can still be employed (as a brake against loss through energetic washing) so that no beads are left in the dead volume.

[0085] In a reaction chamber or a cell culture broth particulate material of interest, or cells, can be held in one or more aggregates in an ultrasound field. Reactants or metabolites in the liquid need to encounter the particulate material to react, or be consumed, and non-specific reagents or products of cellular metabolism need to be removed to allow the process to proceed maximally.

[0086] Typically the particulate materials are held in an aggregate in a chamber and a pump is used to draw liquid over and through the aggregate. In practice, the liquid tends to stream around the clump of particles or cells and acoustic streaming determines how much of the liquid actually reaches the centre of the particle aggregate.

[0087] The ultrasound force is particularly strong in pushing particulate material or cells into a nodal plane. Some of the known examples use a flow perpendicular to the nodal plane to stress the aggregates formed by the ultrasound field. The aggregate of particles is held less strongly in this plane and this imposes a restriction in the dimensions of the

chamber that can be utilised. Until now this has been limited to a volume of around 100 μl which limits the applications that can usually employ this technique. The chambers fabricated for use with ultrasound typically have a gap between transducer (or protective carrier layer on top of the transducer) and the reflector of 1, 2 or 3 half wavelengths. In the case of a 1.5 MHz transducer this equates to a 0.5, 1 or 1.5 mm gap. At these dimensions the liquid flow will exhibit laminar flow properties in the chamber. As laminar flow follows a parabolic pattern, the liquid in the centre of the chamber will have the fastest flow rate, whilst as the liquid is closer to the carrier layer or reflector, the flow will be slower until at the surfaces there is almost no flow at all. This means that the ultrasound force is holding particle aggregates at the position of maximum fluid flow, but where its resistance to movement in the flow is weakest because the strongest force is perpendicular to the nodal plane.

- [0088] By applying the two forces of magnetism and ultrasound standing waves according to this invention on a paramagnetic particle to which materials of interest have been adsorbed provides unexpected improvements in particle handling performance.
- [0089] A sample under investigation is likely to contain two different types of contaminants that could interfere with analysis or reaction. The first is large particles, such as non-desired cells, particularly blood cells. The second is soluble factors that could bind to the particles or otherwise adversely affect the chance of interaction between a ligand and a target molecule.
- [0090] Ultrasound is very effective at addressing the second issue. Without it, the particles alone will still rely upon the relatively slow Brownian motion in the liquid to ensure that ligands encounter their target molecule. Ultrasound stimulates acoustic streaming along at least two axes in a three dimensional space leading to a highly efficient mixing reaction and greatly speeding up the ligand – analyte interaction. This observation has previously been reported in the ability of ultrasound to speed up the reaction and also improve the sensitivity of a latex agglutination slide test for meningitis.
- [0091] However, ultrasound alone is not very good at addressing the first issue, namely removing large contaminating particles or cells from the target particles as the aggregates form on the basis of size and there is insufficient discrimination between cell sizes to be effective. Moreover as particles move into aggregates they can also ‘herd’ or co-entrap smaller particles that would normally not be affected by the ultrasound. Use of magnetism as described in this invention, on the other hand, is very good at pulling paramagnetic particles to the floor or ceiling of the chamber. Since the particles are then located in the slowest part of the parabolic flow in the laminar flow in the chamber the particles can be washed with a high flow rate, thereby rapidly removing the contaminating cells.

- [0092] However, the paramagnetic particles at the floor or ceiling of a chamber can prove resistant to resuspension, as reported earlier. The particles will still be contaminated with soluble factors from the sample but washing is ineffective as they are in the slowest flow of the chamber. By reintroducing an ultrasound standing wave in the chamber the paramagnetic particles are resuspended, and in moving between the floor and the pressure node they mix with the liquid in the chamber, thereby aiding washing. Further they may be washed while held in the pressure node and a continuing magnetic field applied to the chamber makes the paramagnetic particles resistant to being flushed from the chamber by the washing fluid.
- [0093] In this specification the word “chamber” is used to describe any vessel that has an ultrasound transducer associated with it. It may be a chamber as shown in Figure 1, or a straight conduit with no change in diameter between the inlet and outlet, or any other vessel in which the method described may be carried out.
- [0094] The method and apparatus of the invention is applicable to larger chambers than those described in Figures 1 and 2. As discussed above, the size of paramagnetic beads/particles is a compromise between achieving density close to 1.0 and as small a size as possible for mixing and rapid reaction kinetics. Typically, particle sizes of 2.7 μM do not contain sufficient paramagnetic material in the core to respond efficiently to an electromagnet. The aggregation of particles into clumps provides a larger mass of magnetic material, which has been found to respond well to an electromagnet.
- [0095] Also, as the transducer generating the ultrasound standing wave can be driven at a lower voltage in the design described in this invention, a larger volume chamber can be designed without the problems of overheating reported previously.
- [0096] In a further example, therefore, a chamber was constructed with a fill volume of 0.75 ml. A transducer with resonant frequency of 1 MHz (Ferroperm, Denmark) was scored on the top electrode to match the working area of the chamber and fixed to the base of the chamber using conductive epoxy resin. The active transducer as connected to the chamber was 2.5 cm x 2.5 cm. The chamber was milled out of Macor machinable ceramic with the base in contact with the transducer having a thickness of 0.3 mm. The internal height of the chamber was 1.2 mm and the reflector layer was a soda glass double width microscope slide of 1 mm thick. These dimensions produced a chamber with three half-wavelengths within the cavity and therefore three nodal planes developed inside the chamber when driven at a frequency of 1.612 MHz and an amplitude of 200.
- [0097] The larger transducer and the three nodal plane format created hundreds of aggregates of particles in three vertical layers. An electromagnet outside the floor or ceiling of the chamber and driven at an amplitude of 150 and a frequency of 1MHz could draw aggregates to the floor or the ceiling of the chamber once the ultrasound

amplitude was decreased. It was also observed that if the aggregates were drawn to the floor of the chamber, they would settle and spread out slightly and upon increasing the amplitude of the ultrasound the particles at the centre would re-suspend, but particles that had spread out were less affected. The magnet was therefore applied from above to draw the particles to the ceiling of the chamber, where gravity in addition to the ultrasound could be employed to re-suspend particles into the nodal planes.

[0098] **Example 3.**

[0099] Step 1. Switch USW on (Transducer Amplitude=200 F=1.612MHz, Magnet Amplitude=0 F=1MHz);

[0100] Step 2. Fill chamber with bead solution;

[0101] Step 3. Wait 2 to 3 mins for agglomeration to occur (using Lodestar 2.7um beads);

[0102] Step 4. Switch USW off to allow all agglomerates to settle to the bottom (~10s);

[0103] Step 5. USW on to re-agglomerate on the bottom nodal plane;

[0104] Step 6. Switch on magnet (mounted above the glass) to collect agglomerates (Magnet Amplitude = 150);

[0105] Step 7. Flow in next chamber-full of bead solution;

[0106] Step 8. Repeat steps 3-6 as necessary;

[0107] Step 9. Lower USW strength to prevent lateral trapping (Transducer Amplitude = 30);

[0108] Step 10. Pump out beads (or alternatively a 2nd magnet was used to capture the paramagnetic beads near the exit port).

[0109] Key Device Parameters for Example 3 :

Main chamber volume 750µL

Agglomeration Amplitude (on signal generator) 200

Flow out Amplitude (less trapping) 30

Operating frequency (nominal) 1.612MHz

Magnet activation amplitude 150

Magnet activation frequency 1MHz

Maximum Flow rate for uniform flow 3mL/min

[0110] In a further development of the invention, a disposable separation apparatus chamber is used.

[0111] Ultrasound chambers designed to agglomerate particles are typically fabricated with a rigid connection between the transducer and the floor of the chamber. Such chambers are usually fabricated out of rigid materials such as steel or ceramics such as Macor. Research has shown that sound energy propagates through rigid materials with lower losses than through flexible materials such as plastics. The separation apparatus of this invention was specifically directed for use in connection with diagnostic processes, such as detection of Tuberculosis and other disease-causing organisms. Tests for these

applications need to be safe, but also disposable to ensure no cross contamination between samples. The manufacturing prices for the transducers and rigid chambers are too high compared to the prices paid for disposable tests and a key purpose of the invention is to allow for the separation apparatus to be fabricated out of low cost polymers (e.g. injection moulding) which requires a different mechanism of coupling to the transducer (the reusable component)..

[0112] A disposable chamber is provided to be used as part of an instrument utilising the method of this invention.

[0113] As described above, the interplay of the magnetic and ultrasound fields creates a holding power stronger than either alone. Consequently it has been found that the acoustic chamber can still function to effect separation of particles with less efficient transfer of acoustic energy into the fluid in the chamber. The floor of the chamber is designed to minimise losses in the transfer of acoustic energy and laminate films have proved to be usable.

[0114] To ensure coupling to the transducer, one of several methods can be employed. The first involves adding a drop of liquid to ensure mechanical contact via surface tension between the faces of the transducer and the chamber base. Machine oils, glycerol or other liquids are suitable. The second method illustrated in Figures 3 and 4 uses low pressure between the transducer and the base of the chamber, which was achieved by creating holes through the transducer and applying a pump to withdraw any air between the two faces. As a further alternative, if thin film plastic as described below is used, the transducer can simply be pushed on to the plastic and held in place by deformation of the plastic.

[0115] In Figures 3 and 4 the separation apparatus 50 comprises a first chamber 52, reflector wall 53, side wall 59 and a thin film cover 62 together with an inlet 54 and outlet 56. The outlet 56 has a small collection chamber 58 formed therein. Inlet 54 and outlet 56 have shaped ends 55 and 57 respectively to which external tubing from other apparatus may be attached. The chamber 52 with reflector wall 53, inlet 54 and outlet 56 are a single transparent acrylic moulding with an open aperture closed by the film 62, these items form a single disposable unit.

[0116] Chamber 52 operates with a transducer 60 with resonant frequency of 1 MHz (Ferroperm, Denmark) mounted above the chamber 52 and coupled to the chamber by a sheet of thermal shrink film 62 (Frostking, USA). Thin film 62 forms the carrier layer of the chamber. The transducer was scored on the top electrode to match the working area of the chamber (2.5 x 2.5 cm) and coupled to the film 62 using a drop of glycerol, or alternatively using a partial vacuum created by applying a vacuum pump V (not shown) through housing 64 (the transducer is not part of the disposal apparatus and is reusable). To aid coupling holes 66 are formed in the transducer 60. The main

body of the chamber 52 was milled from acrylic material and the open top covered by the sheet of thermal shrink film 62. This film had a thickness of 20 μm . The internal height of the chamber was 1.05 mm and the reflector layer was 0.35 mm thick acrylic. These dimensions produced a chamber with three half-wavelengths and therefore three nodal planes were present in the chamber when driven at a frequency of 1.740 MHz. The chamber and inlet and outlet are supported in an acrylic support 63 comprising upper member 64 and lower member 68, upper member 64 pivotally mounted on a pivot 74 on lower member 68. The lower member 68 has supporting legs 71 either ends to be received on a stand 70. The upper and lower members 64 and 67 have moulded surfaces to receive the chamber moulding and to support it in position in the support 63.

- [0117] Upper member 64 has a recess 65 which is connectable to a vacuum pump V (not shown), to reduce the pressure within the recess 65. When this occurs thin film 62 clings tightly to the transducer 60, thus holding it in place above the chamber 52.
- [0118] The chamber's side walls 59 support the chamber in a recess 67 formed in lower member 64. The recess 67 has an indentation 69 below the reflector wall of the chamber 53 creating an air gap below the base of the chamber to reduce transmission of vibrations from the chamber to member 68 when the transducer 60 is operating. The separation apparatus 50 is mounted on a stand 70 having a cavity 72 in which control apparatus is conveniently mounted.
- [0119] One or more magnets 76 are mounted beneath the lower member 68. The magnets may be permanent magnets, electromagnets or solid state magnets, the latter of the kind which when orientated in one direction produce a magnetic field and when turned by 90° have no field. In this particular case, there is a single electromagnet 76 mounted on a screw 78 passing through bearings 82 and 84 in legs 71 of the lower member 68. The position of the magnet is controlled by a step motor 80. The magnet can be placed in various positions below the chamber 52, progressively moving from right to left in Figure 4 to a final position below the collection chamber 58. In the final position the magnet is used to collect the paramagnetic particles from the chamber after the acoustic transducers have been turned off before all the particles are flushed from the chamber out of outlet 56 for further analysis.
- [0120] The pressure amplitude inside the device was measured using the force-balance technique as described by S. P. Martin, R. J. Townsend, L. A. Kuznetsova, K. A. J. Borthwick, M. Hill, M. B. McDonnell and W. T. Coakley, Biosens Bioelectron, 2005, 21, 758. To measure the strength of the node, a 10 μm diameter polystyrene bead was held in the acoustic field, and the drive voltage steadily reduced until the bead began to sediment. Thus the voltage at which the acoustic force was exactly balanced by the gravitational force was determined.

- [0121] An acoustic pressure amplitude of 112kPa (+20%) was estimated at the operational drive voltage of 4Vpp for the disposable chamber described in figures 3 and 4. The original ceramic chamber described in Example 3 above was also tested in comparison and found to exert a similar pressure amplitude of 120kPa. Although the materials in the disposable device are lossier than those of the original the reduced thickness of the carrier and reflector layers enables this disposable device to behave as well as the original reusable one.
- [0122] Additional experiments also demonstrated that aggregate formation was effective over a wide range of frequencies when using a thin polymer film as the carrier layer chamber, whereas a solid steel or Makor carrier layer would only be active at a precise frequency. The polymeric film is also easier to use during the manufacture of the product, by providing greater tolerance in performance than in the all solid chambers.
- [0123] Although the device in Figures 3 and 4 has been described with the acoustic transducer 60 mounted on a thin film above the chamber 52, with the magnet 76 below, the chamber has also been designed to be the other way up, with the transducer 60 below the chamber and thin film 62 forming the lower wall of the chamber. The moulded shapes of members 64 and 68 would need to be different in order to accommodate this different orientation with features of the lower member 68 above moulded in the upper member and vice versa.

Claims

- [0001] A method of processing of a sample mixed with paramagnetic particles characterised in that it includes forming an ultrasound standing wave to drive the paramagnetic particles into one or more pressure nodes and exposing the sample mixed with paramagnetic particles to a pulling force to draw the paramagnetic particles.
- [0002] A method of processing a sample according to claim 1 characterised in that it comprises the steps of mixing the sample with paramagnetic particles with surfaces that capture materials of interest, introducing at least part of the sample material in a fluid into a chamber, establishing at least one pressure node in the chamber by means of an acoustic standing wave in the fluid, aggregating the sample at the node, reducing the strength of or eliminating the acoustic standing wave and subjecting the paramagnetic particles to a pulling force to draw them towards a wall of the chamber, and re-establishing the acoustic standing wave to drive the paramagnetic particles back into pressure nodes.
- [0003] A method according to claim 1 or 2 characterised in that the pulling force pulls the paramagnetic particles towards a wall of the chamber when the strength of the acoustic standing wave is reduced or the acoustic standing wave eliminated.
- [0004] A method according to claim 3 characterised in that it includes the step of washing the paramagnetic particles while they are held by the pulling force at a wall of the chamber.
- [0005] A method according to claim 4 characterised in that it includes the step of passing a laminar fluid flow over the paramagnetic particles while they are held by the pulling force at the wall.
- [0006] A method according to any preceding claim characterised in that including the step of reducing or eliminating the acoustic standing wave and re-establishing the acoustic standing wave on a plurality of occasions.
- [0007] A method according to any preceding claim characterised in that the pulling force is exerted by gravity.
- [0008] A method according to any preceding claim characterised in that the pulling force is exerted by a magnetic field.
- [0009] A method according to claim 8 characterised in that it additionally includes the step of applying a magnetic field to the chamber as the sample is admitted to the chamber.
- [0010] A method according to claim 8 or 9 characterised in that it includes the step of increasing the magnetic field while particles are held in an acoustic wave of constant strength.

- [0011] A method according to claim 8 or 9 including the step of decreasing or increasing the magnetic field while particles are held in an acoustic wave of constant strength.
- [0012] A method according to claim 8 characterised in that a magnetic field is continuously applied to the chamber while any paramagnetic materials are present in the chamber.
- [0013] A method according to any one of claims 9 to 12 characterised in that it includes the step of forming aggregates of greater mass than single particles, and thus increasing the effect of a magnetic field.
- [0014] A method according any preceding claims characterised in that it includes the step of washing the paramagnetic particles while held in an acoustic standing wave.
- [0015] A method according to any one of the preceding claims including the step of gathering the paramagnetic particles in a magnetic field after the particles have passed though a chamber in which pressure nodes may be formed.
- [0016] A method according to any one of the preceding claims characterised in that the sample contains biological particles.
- [0017] A method according to claim 16 characterised in that the paramagnetic particles have surfaces that will capture biological particles.
- [0018] A method according to claim 16 or 17 in which the biological particles are bacteria.
- [0019] A method according to any preceding claim characterised in that the method forms part of a process to extract biological cells from inhibitors.
- [0020] A method according to any preceding claim characterised in that the sample is one being tested for Mycobacterium tuberculosis.
- [0021] A method according to any preceding claim characterised in that the method includes disposing of the chamber after processing each sample.
- [0022] Separation apparatus for use in the analysis of biological samples characterised in that said apparatus comprises at least a first chamber, an inlet and an outlet to said chamber, at least one acoustic transducer capable of creating at least one pressure node in a fluid contained in the chamber and mounted against a wall of said chamber, and one or more magnets associated with the apparatus to attract paramagnetic materials contained in a sample.
- [0023] Separation apparatus according to claim 22 characterised in that a magnet is positioned on the outside of a wall of said first chamber.
- [0024] Separation apparatus according to claim 23 in which said wall is acoustic reflecting wall of the chamber.
- [0025] Separation apparatus according to any one of claims 22 to 24 characterised in

- that one wall of the chamber comprises a flexible thin film material.
- [0026] Separation apparatus according to claim 25 characterised in that said acoustic transducer is affixed to the outside surface (that surface not facing into the chamber) of the flexible thin film material.
- [0027] Separation apparatus according to claim 26 characterised in that the thin film material is about 20µm thick.
- [0028] Separation apparatus according to claim 26 or 27 characterised in that the acoustic traducer is coupled to the thin film material without adhesive.
- [0029] Separation apparatus according to any one of claims 25 to 28 characterised in that the apparatus is operable over a range of frequencies to form aggregates.
- [0030] Separation apparatus according to claim 28 characterised in that the outside surface of said thin film material is under reduced pressure.
- [0031] Separation apparatus according to any one of claims 25 to 30 characterised in that a magnet is placed against the outlet to collect paramagnetic particles in its associated magnetic field after the paramagnetic particles have passed though the chamber.
- [0032] Separation apparatus according to claim 31 characterised in that the outlet has a second chamber and a magnet is positioned against the wall of said chamber.
- [0033] Separation apparatus according to any one of claims 22 to 32 characterised in that the chamber and the inlet and outlet are disposable and are mountable in a frame.
- [0034] A chamber for use in the analysis of biological samples characterised in that said chamber comprises a one piece moulding with inlet and outlets, a side wall and an acoustic reflecting wall, said side wall with a thin film wall disposed opposite said acoustic reflecting wall and attached to the side wall.
- [0035] A chamber according to claim 34 characterised said moulding consists of transparent acrylic plastic material.
- [0036] A chamber according to claim 34 or 35 characterised in that the thin film material is about 20µm thick.
- [0037] A chamber according to one of claims 34 to 37 characterised in that an acoustic transducer is attached to the outside surface of the thin film without adhesive.
- [0038] A chamber according to any one of claims 34 to 37 characterised in that the apparatus is operable over a range of frequencies to form the aggregate.
- [0039] A chamber according to any one of claims 34 to 38 characterised in that a magnets is placed against the outlet to collect paramagnetic particles in its as-sociated magnetic field after the paramagnetic particles have passed though the chamber.
- [0040] A chamber according to any one of claims 34 to 39 characterised in that the

outlet has a second chamber and a magnet may be placed against the outlet is positioned against the wall of said chamber.

- [0041] A chamber according to any one of claims 34 to 40 characterised in that the acoustic transmitter is attached to the thin wall without adhesive.
- [0042] A chamber according to claims 41 characterised in that the acoustic transmitter is attached to the thin wall by plastic deformation of the thin wall
- [0043] A chamber according to claim 42 characterised the acoustic transmitter is attached to the thin wall by applying a reduced pressure to the outside surface of the thin wall.
- [0044] A chamber according to claim 41 characterised the acoustic transmitter is attached to the thin wall by a liquid coupling layer.
- [0045] A chamber according to claim 44 characterised in that the liquid coupling layer is glycerol.
- [0046] A chamber according to any one of claims 34 to 45 characterised in that one or more magnets are mounted adjacent to but outside the reflecting wall.
- [0047] A chamber according any one of claim 34 to 46 characterised in that it is mounted in a frame.
- [0048] A chamber according to claim 47 characterised in that an air gap is provided between the reflecting wall of the chamber and a frame member.
- [0049] A chamber according to claim 46, 47 or 48 characterised in that the one or magnets are above the chamber and the thin film wall is the lower wall.
- [0050] A chamber according to anyone of claims 34 to 49 characterised in that said one or more magnets may apply magnetic fields to different parts of the chamber at different times.

[Fig.]

Fig.1.

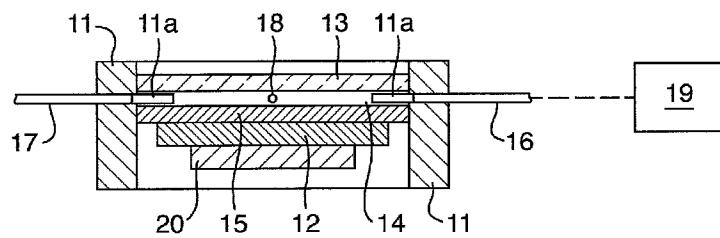


Fig.2.

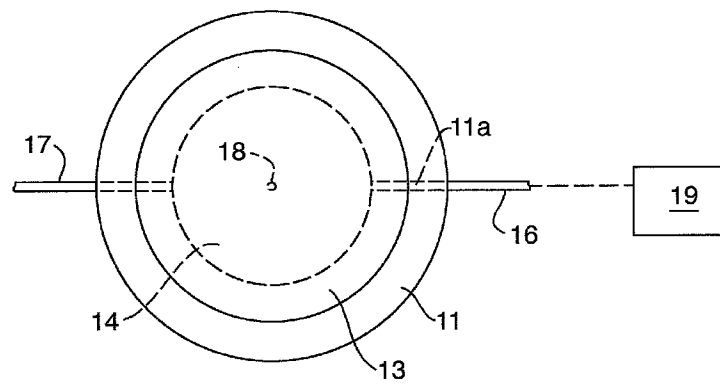
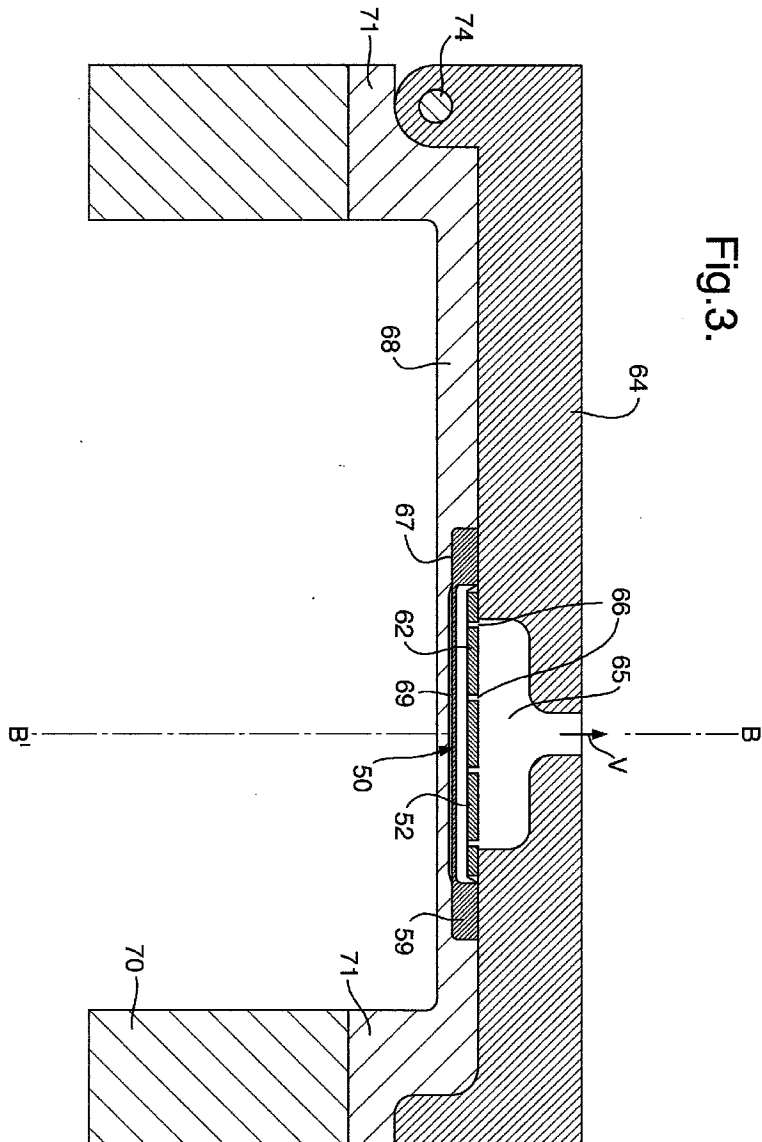


Fig. 3.



[Fig.]

