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(54) Title: COMPOSITIONS AND METHODS FOR MODULATING CELLULAR MEMBRANE-MEDIATED INTRACELLULAR SIGNAL TRANSDUCTION

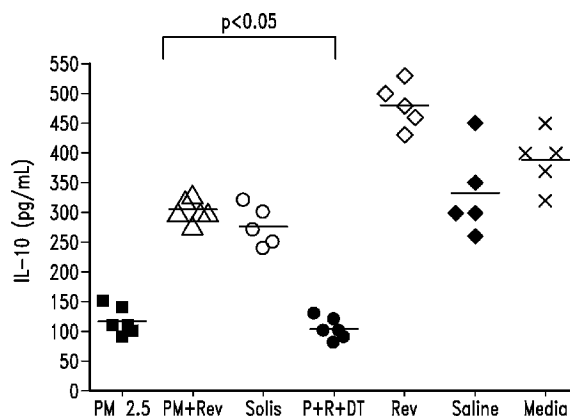


Fig. 77

(57) Abstract: Particular aspects of the present invention provide compositions and methods for modulating intracellular signal transduction, including modulation of at least one of membrane structure, membrane potential or membrane conductivity, membrane proteins or receptors, ion channels, and calcium dependant cellular messaging systems, comprising use of the inventive electrokinetically generated solutions to impart electrochemical and/or conformational changes in membranous structures (e.g., membrane and/or membrane proteins, receptors or other components) including but not limited to G-protein coupled receptors (GPCRs) and/or G-proteins, and intracellular junctions. In particular embodiments, the electrokinetically generated solutions or compositions are gas-enriched and/or comprise other therapeutic agents. Other embodiments include particular therapeutic methods comprising administration or formulations of the therapeutic compositions, and in various combination treatments.

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COMPOSITIONS AND METHODS FOR MODULATING CELLULAR MEMBRANE-MEDIATED INTRACELLULAR SIGNAL TRANSDUCTION

FIELD OF THE INVENTION

Certain embodiments disclosed herein relate to the field of regulating or modulating intracellular signal transduction by modulation of at least one of cellular membranes, membrane potential, membrane proteins such as membrane receptors, including but not limited to G protein coupled receptors, and intercellular junctions (e.g., tight junctions, gap junctions, zona adherins and desmosomes). Particular aspects relate to modulating (e.g., treating or preventing) at least one disease or condition or symptom thereof associated with cellular membrane-mediated signal transduction (e.g., mediated by membrane receptors), including signaling mediated by G protein coupled receptors by administering a therapeutic composition comprising at least one electrokinetically generated fluid (including gas-enriched (e.g., oxygen enriched) electrokinetically generated fluids) as disclosed herein.

BACKGROUND

Many factors influence or are associated with cellular membrane-mediated responses, including, for example, gene transcription, and protein production. Interactions with a cell surface or membrane or constituent thereof (e.g., membrane-associated proteins and/or receptors) results in cascades of events (signal transduction pathways) reflecting the type, specificity, affinity/intensity, and duration of the interaction with the membrane or membrane constituent. Many diseases and disorders are associated with aberrant cellular events that are associated with altered or aberrant membrane interactions. Exemplary factors that influence membrane responses and subsequent signal transduction and cellular reactions include transmembrane proteins (such as G protein coupled receptors), cellular membrane junctions (e.g., tight junctions, gap junctions, zona adherins and desmosomes), as well as membrane potential and/or conductance, and Cell Adhesion Molecules (CAMs) located on the cell surface and involved with the binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion.

GPCRS. There are several known families of membrane bound receptors, transmembrane receptors, or membrane associated receptors. Guanine-nucleotide binding protein coupled receptors, or G protein coupled receptors (GPCRs), also known as seven transmembrane receptors, 7TM receptors, heptahelical receptors, and G protein linked receptors (GPLR), comprise a large protein family of transmembrane receptors that sense molecules outside of the cell and activate and/or mediate cytoplasmic signal transduction responses leading to cellular responses. For example, particular G protein coupled receptors are activated by light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters that vary in size from small molecules to peptides to large proteins.

Based on sequence homology and functional similarity, GPCRs can be classified into 6 groups: class A/1 comprise rhodopsin like molecules; class B/2 comprise secretin receptor family molecules; class C/3 comprise metabotropic glutamate/pheromone receptors; class D/4 comprise fungal mating pheromone receptors; class E/5 are cyclic AMP receptors; and class F/6 comprise Frizzled/Smoothed receptors. The extracellular components of the GPCR contain two highly-conserved cysteine residues that form disulfide bonds to stabilize the receptor structure.

GPCRs are set within the plasma membrane, and interact with a scaffolding of cytoplasmic proteins, primarily G proteins (α , β and γ). The binding of the ligand to its receptor triggers an activation of the complex, which in turn mediates the activity of other cell effectors. Two primary secondary messengers can be detected following activation of a GPCR. The first concerns the receptors coupled to Gas and Gai proteins, and modulates the activity of Adenylate Cyclase (AC), which catalyses cAMP production. The second, mediated by the G α_q subunit, triggers phospholipase C activity, which converts phosphatidylinositol-4,5-bisphosphate (PIP₂) into inositol-1,3,5-triphosphate (IP₃). The heterotrimeric G proteins, consisting of α , β and γ subunits, couple ligand-bound seven transmembrane domain receptors (GPCRs or G-protein coupled receptors) to the regulation of effector proteins and production of intracellular second messengers such as cAMP, cGMP, and Ca^{2+} . G protein signaling mediates the perception of environmental cues in all higher eukaryotic organisms, including yeast, Dictyostelium, plants, and animals. Agonist-bound sensory receptors catalyze the exchange of GTP for GDP on the surface of the G α subunit to initiate intracellular responses to extracellular signals. Intracellular signaling is mediated

through various effector enzymes, including cGMP phosphodiesterase, phospholipase C, adenylate cyclase, etc. (see Kinnamon & Margolskee, 1996, *Curr. Opin. Neurobiol.* 6: 506-513). Most effector proteins interact with the G_α , although $G\beta\gamma$ subunits also contribute to the specificity of receptor-G protein coupling (Xu et al., 1998, *J. Biol. Chem.* 273(42): 27275-79).

The G protein α subunits are grouped into four families, G_{α_s} , G_{α_i} , G_{α_q} , and $G_{\alpha_{12}}$ according to their sequence homologies and functional similarities. The G_{α_q} family members couple a large group of GPCRs to phospholipase C. Activation of G_{α_q} coupled GPCRs induces intracellular calcium release and the capacitative calcium entry from extracellular space. The consequential increase of cytosolic calcium concentration can be effectively detected by using synthetic or genetically-engineered fluorescent calcium indicators, bioluminescent calcium indicators, calcium-activated ion currents, and by monitoring calcium-regulated gene transcription. Assays based on such calcium readout are available in high-throughput screening (HTS) format.

The G_{α_q} (G_q) class includes four proteins expressed in mammals, called G_{α_q} , $G_{\alpha_{11}}$, $G_{\alpha_{14}}$, and $G_{\alpha_{15}}$ (in mice, $G_{\alpha_{16}}$ in humans). Whereas orthologs of these subunits are highly conserved across species (99, 97, 96 and 85% identity, respectively), paralogs of these subunits (expressed in the same species) are not as conserved. This suggests that each type of subunit in the G_q class has a distinct function, however, when transfected into Sf9 cells, the subunits stimulated phospholipase C with similar potency and showed similar activities (Nakamura et al., 1995, *J. Biol. Chem.* 270: 6246-6253). Xu and colleagues subsequently showed by gene knockouts in mice that G_{α_q} subunits promiscuously couple to several different receptors in various cell types (1998, *J. Biol. Chem.* 273(42): 27275).

Current methods employed in GPCR screening programs measure various cellular signaling events. The most widely used include measuring the formation of cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP3), and intracellular Ca^{2+} mobilization. Generally, cAMP measurement provides useful data when screening receptors known to couple to G_i/o or G_s , whereas Ca^{2+} mobilization or IP3 formation assays are most useful for measuring activation of $G_q/11$ -coupled receptors.

GPCRs are involved in a wide variety of physiological processes, including but not limited to vision (e.g. rhodopsin converts 11-cis-retinal to all-trans-retinal), sense of smell (e.g. olfactory receptors and pheromones), behavioral and mood regulation (e.g.

neurotransmitters), regulation of the immune system activity and inflammation (e.g. chemokines), autonomic nervous system transmission (e.g. blood pressure, heart rate, digestive processes), and cell density sensing.

The majority of prescribed drugs target either activation of GPCRs or their downstream signals, particularly for drugs used in the management of airway diseases such as asthma and other airway smooth muscle conditions. Billington and Penn, *Respiratory Res.* 4:2 (2003).

Bradykinin, for example, is a physiologically and pharmacologically active peptide of the kinin group of proteins. The Bradykinin receptors are members of the G protein-coupled receptor family. Bradykinin protein is composed of 9 amino acids (polypeptide comprising 9 aa residues: Arginine- Proline- Proline- Glycine- Phenylalanine- Serine- Proline- Phenylalanine- Arginine. Bradykinin is generated by proteolytic cleavage of its precursor, kininogen, by the enzyme kallikrein. Angiotensin-Converting Enzyme (ACE), aminopeptidase P (APP), and carboxypeptidase N (CPN) all degrade Bradykinin.

Bradykinin acts as a potent endothelium-dependent vasodilator, causing contraction of non-vascular smooth muscle, an increase in vascular permeability and is also involved in pain mechanisms. Overactivation of Bradykinin is thought to play a role in certain rare diseases, such as hereditary angioedema (also called hereditary antio-neurotic edema).

The Bradykinin B1 receptor is expressed as a consequence of tissue injury, and presumably plays a role in pain mechanisms, as well as inflammation. The Bradykinin B2 receptor is constitutively active and may participate in vasodilation.

Tight Junctions. Tight junctions, also called occluding junctions or zonula occludens, are composed of multimeric protein complexes of different types of proteins that interact with each other. Tight junctions are required for epithelial barrier formation and assist in regulating signaling mechanisms for proliferation and differentiation by preventing ion and other molecules from passing across the membrane(s), holding cells together, and blocking the movement of integral membrane proteins between the apical and basolateral surfaces of the cell. Anderson et al., *Curr. Opin. Cell Biol.* 16:140-145 (2004); Schneeberger and Lynch, *Am. J. Physiol.* 286:C1213-28 (2004). In addition to

tight junctions, other cellular junctions include gap junctions, zona adherens and desmosomes.

Membrane Potential. Membrane potential (also called transmembrane potential or transmembrane potential difference or transmembrane potential gradient) is the electrical potential difference (measured by voltage) across a cell's plasma membrane. Membrane potential arises from the action of ion transporters embedded in the membrane which maintain viable ion concentrations inside the cell. The term "membrane potential" is sometimes used interchangeably with cell potential but is applicable to any lipid bilayer or membrane. The typical membrane potential of a cell arises from the separation of ions (e.g., sodium, calcium, potassium) from intracellular immobile anions across the membrane of the cell. This separation results from a concentration gradient of potassium ions by pumps or transporters. While there is an electric potential across the membrane due to charge separation, there is no actual measurable difference in the global concentration of positive and negative ions across the membrane. Thus, there is no measurable charge excess on either side. Cell membranes are typically permeable to only a subset of ionic species, including but not limited to potassium ions, chloride ions, bicarbonate ions, calcium ions and others.

SUMMARY OF THE INVENTION

Particular aspects of the present invention provide compositions and methods for modulating intracellular signal transduction, including modulation of at least one of membrane structure, membrane potential or membrane conductivity, membrane proteins or receptors, ion channels, and calcium dependant cellular messaging systems, comprising use of the inventive electrokinetically generated solutions to impart electrochemical and/or conformational changes in membranous structures (e.g., membrane and/or membrane proteins, receptors or other components) including but not limited to GPCRs and/or g-proteins. According to additional aspects, these effects modulate gene expression, and may persist, dependant, for example, on the half lives of the individual messaging components, etc.

Particular aspects provide methods for modulating intracellular signal transduction, comprising contacting at least one cell having a membrane and membrane components with an electrokinetically altered aqueous fluid, the electrokinetically altered aqueous fluid suitable to alter cellular membrane structure or

function sufficient to provide for modulation of intracellular signal transduction. In particular aspects, alteration of the electrokinetically altered aqueous fluid comprises exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects, where, in certain embodiments, exposure to the localized electrokinetic effects comprises exposure to at least one of voltage pulses and current pulses and/or exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects, comprises exposure of the fluid to electrokinetic effect-inducing structural features of a device used to generate the fluid.

In certain embodiments, altering cellular membrane structure or function comprises altering of a conformation, ligand binding activity, or a catalytic activity of a membrane associated protein. In certain aspects, the membrane associated protein comprises at least one selected from the group consisting of receptors, transmembrane receptors, ion channel proteins, intracellular attachment proteins, cellular adhesion proteins, integrins, etc. In particular aspects, the transmembrane receptor comprises a G-Protein Coupled Receptor (GPCR). In certain embodiments, the G-Protein Coupled Receptor (GPCR) interacts with a G protein a subunit (e.g., at least one selected from the group consisting of G_{α_s} , G_{α_i} , G_{α_q} , and $G_{\alpha_{12}}$). In particular aspect, the G protein a subunit is G_{α_q} .

In certain aspects, altering cellular membrane structure or function comprises altering membrane conductivity or membrane potential. In certain embodiments, modulation of intracellular signal transduction comprises modulation of a calcium dependant cellular messaging pathway or system (e.g., comprises modulation of at least one of phospholipase C activity and adenylate cyclase (AC) activity).

In certain aspects, modulation of intracellular signal transduction comprises modulation of intracellular signal transduction associated with at least one condition or symptom selected from the group consisting of inflammation, asthma, neurodegeneration, abnormalities of the brain, central nervous system disruption or degradation, Alzheimer's Disease, aging, developmental abnormalities of bone, altered bone growth, hormone resistance, pseudohypoparathyroidism, hormone hypersecretion, McCune-Albright syndrome, retinal disorders, endocrine disorders, metabolic disorders, developmental disorders, alterations in pigmentation of the skin, premature sexual development, psychological maladies, lung constriction, bronchial

constriction, alveolar constriction, metabolic symptoms, insulin resistance, and retinal disruption or degradation.

Certain aspects of the above methods comprise contacting a cell network or layer, and further comprises modulation of an intercellular junction therein (e.g., at least one selected from the group consisting of tight junctions, gap junctions, zona adherens and desmosomes). In certain embodiments, the cell network or layer comprises at least one selected from the group consisting of pulmonary epithelium, bronchial epithelium, intestinal epithelium, and corneal epithelium.

In certain aspects of the method, the electrokinetically altered aqueous fluid is oxygenated, wherein the oxygen in the fluid is present in an amount of at least 15, ppm, at least 25 ppm, at least 30 ppm, at least 40 ppm, at least 50 ppm, or at least 60 ppm oxygen at atmospheric pressure. In certain embodiments, the electrokinetically altered aqueous fluid comprises at least one of solvated electrons, and electrokinetically modified or charged oxygen species (e.g., wherein the solvated electrons and/or electrokinetically modified or charged oxygen species are present in an amount of at least 0.01 ppm, at least 0.1 ppm, at least 0.5 ppm, at least 1 ppm, at least 3 ppm, at least 5 ppm, at least 7 ppm, at least 10 ppm, at least 15 ppm, or at least 20 ppm). In particular aspects, the electrokinetically altered oxygenated aqueous fluid comprises solvated electrons stabilized by molecular oxygen.

In certain aspects, the ability to alter cellular membrane structure or function sufficient to provide for modulation of intracellular signal transduction persists for at least two, at least three, at least four, at least five, at least 6 months in a closed container (e.g., closed gas-tight container).

Additional aspects provide a method for modulating cellular membrane conductivity, comprising contacting at least one cell having a membrane and membrane components with an electrokinetically altered aqueous fluid, the electrokinetically altered aqueous fluid suitable to alter cellular membrane structure or function sufficient to provide for modulation of cellular membrane conductivity. In certain aspects, modulating cellular membrane conductivity, comprises modulating whole-cell conductance. In particular embodiments, modulating whole-cell conductance, comprises modulating at least one voltage-dependent contribution of the whole-cell conductance. In certain aspects, alteration of the electrokinetically altered aqueous fluid comprises exposure of the fluid to hydrodynamically-induced, localized

electrokinetic effects (e.g., comprising exposure to at least one of voltage pulses and current pulses). In particular aspects, exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects, comprises exposure of the fluid to electrokinetic effect-inducing structural features of a device used to generate the fluid. In particular embodiments, modulating cellular membrane conductivity comprises modulation of at least one selected from the group consisting of membrane receptors, transmembrane receptors, ion channel proteins, intracellular attachment proteins, cellular adhesion proteins and integrins. In certain aspects, modulating cellular membrane conductivity comprises modulation of ion channel proteins.

In preferred aspects of the above-described methods, the cell is a mammalian cell, and most preferably a human cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a partial cross-section, partial block diagram of a prior art mixing device.

Figure 2 is block diagram of an exemplary embodiment of a mixing device.

Figure 3 is an illustration of an exemplary system for delivering a first material to the mixing device of Figure 2.

Figure 4 is a fragmentary partial cross-sectional view of a top portion of the mixing device of Figure 2.

Figure 5 is a fragmentary cross-sectional view of a first side portion of the mixing device of Figure 2.

Figure 6 is a fragmentary cross-sectional view of a second side portion of the mixing device of Figure 2.

Figure 7 is a fragmentary cross-sectional view of a side portion of the mixing device of Figure 2 located between the first side portion of Figure 5 and the second side portion of Figure 6.

Figure 8 is a perspective view of a rotor and a stator of the mixing device of Figure 2.

Figure 9 is a perspective view of an inside of a first chamber of the mixing device of Figure 2.

Figure 10 is a fragmentary cross-sectional view of the inside of a first chamber of the mixing device of Figure 2 including an alternate embodiment of the pump 410.

Figure 11 is a perspective view of an inside of a second chamber of the mixing device of Figure 2.

Figure 12 is a fragmentary cross-sectional view of a side portion of an alternate embodiment of the mixing device.

Figure 13 is a perspective view of an alternate embodiment of a central section of the housing for use with an alternate embodiment of the mixing device.

Figure 14 is a fragmentary cross-sectional view of an alternate embodiment of a bearing housing for use with an alternate embodiment of the mixing device.

Figure 15 is a cross-sectional view of the mixing chamber of the mixing device of Figure 2 taken through a plane orthogonal to the axis of rotation depicting a rotary flow pattern caused by cavitation bubbles when a through-hole of the rotor approaches (but is not aligned with) an aperture of the stator.

Figure 16 is a cross-sectional view of the mixing chamber of the mixing device of Figure 2 taken through a plane orthogonal to the axis of rotation depicting a rotary flow pattern caused by cavitation bubbles when the through-hole of the rotor is aligned with the aperture of the stator.

Figure 17 is a cross-sectional view of the mixing chamber of the mixing device of Figure 2 taken through a plane orthogonal to the axis of rotation depicting a rotary flow pattern caused by cavitation bubbles when a through-hole of the rotor that was previously aligned with the aperture of the stator is no longer aligned therewith.

Figure 18 is a side view of an alternate embodiment of a rotor.

Figure 19 is an enlarged fragmentary cross-sectional view taken through a plane orthogonal to an axis of rotation of the rotor depicting an alternate configuration of through-holes formed in the rotor and through-holes formed in the stator.

Figure 20 is an enlarged fragmentary cross-sectional view taken through a plane passing through and extending along the axis of rotation of the rotor depicting a configuration of through-holes formed in the rotor and through-holes formed in the stator.

Figure 21 is an enlarged fragmentary cross-sectional view taken through a plane passing through and extending along the axis of rotation of the rotor depicting an alternate offset configuration of through-holes formed in the rotor and through-holes formed in the stator.

Figure 22 is an illustration of a shape that may be used to construct the through-holes of the rotor and/or the apertures of the stator.

Figure 23 is an illustration of a shape that may be used to construct the through-holes of the rotor and/or the apertures of the stator.

Figure 24 is an illustration of a shape that may be used to construct the through-holes of the rotor and/or the apertures of the stator.

Figure 25 is an illustration of a shape that may be used to construct the through-holes of the rotor and/or the apertures of the stator.

Figure 26 is an illustration of an electrical double layer ("EDL") formed near a surface.

Figure 27 is a perspective view of a model of the inside of the mixing chamber.

Figure 28 is a cross-sectional view of the model of Figure 27.

Figure 29 is an illustration of an experimental setup.

Figure 30 illustrates dissolved oxygen levels in water processed with oxygen in the mixing device of Figure 2 and stored in a 500 ml thin walled plastic bottle and a 1,000 ml glass bottle each capped at 65° Fahrenheit.

Figure 31 illustrates dissolved oxygen levels in water processed with oxygen in the mixing device of Figure 2 and stored in a 500 ml plastic thin walled bottle and a 1,000 ml glass bottle both refrigerated at 39° Fahrenheit.

Figure 32 illustrates the dissolved oxygen retention of a 500 ml beverage fluid processed with oxygen in the mixing device of Figure 2.

Figure 33 illustrates the dissolved oxygen retention of a 500 ml braun balanced salt solution processed with oxygen in the mixing device of Figure 2.

Figure 34 illustrates a further experiment wherein the mixing device of Figure 2 is used to sparge oxygen from water by processing the water with nitrogen in the mixing device of Figure 2.

Figure 35 illustrates the sparging of oxygen from water by the mixing device of Figure 2 at standard temperature and pressure.

Figure 36 is an illustration of an exemplary nanocage.

Figure 37A and B illustrate Rayleigh scattering effects of an oxygen-enriched fluid;

Figure 38 illustrates the cytokine profile of a mitogenic assay in the presence of a gas-enriched fluid and deionized control fluid; and

Figure 39 illustrates the difference in the growth rates of *Pseudomonas* bacteria at various dissolved oxygen saturation ratios.

Figures 40A and 40B illustrate *in vitro* healing of wounds using an oxygen-

enriched cell culture media and a non-gas-enriched media.

Figures 41A through 41F show histological cross-sections of dermal and epidermal *in vivo* wound healing.

Figure 42 illustrates the expression of Hale's stain in treated and control healing wounds, used to detect acid mucopolysaccharides, such as hyaluronic acid;

Figure 43 illustrates the expression of von Willebrand's Factor stain used to detect angiogenesis in treated and control healing wounds;

Figure 44 illustrates the detection of Luna's stain used to detect elastin in treated and control healing wounds;

Figure 45 illustrates the number of mast cells per visual field for treated and control healing wounds;

Figure 46 illustrates the percentage of dead cells at separate time points in a corneal fibroblast assay using inventive gas-enriched culture media and control culture media,

Figure 47 illustrates the shelf life of the inventive gas-enriched fluid in a polymer pouch;

Figure 48 illustrates the results of contacting splenocytes with MOG in the presence of pressurized pot oxygenated fluid (1), inventive gas-enriched fluid (2), or control deionized fluid (3).

Figures 49-58 show the results of whole blood sample evaluations of cytokines.

Figures 59-68 show the corresponding cytokine results of bronchoalveolar lavage fluid (BAL) sample evaluations.

Figures 69-75 shows studies where the Bradykinin B2 membrane receptor was immobilized onto aminopropylsilane (APS) biosensor. The Sample plate set up was as designated in Figure 69 and the binding of Bradykinin to the immobilized receptor was assessed according to the sample set up as designated in Figure 71. Results of Bradykinin binding are shown in Figure 72. Bradykinin binding to the receptor was further titrated according to the set-up as designated in Figure 73. As indicated in Figure 74, Bradykinin binding to the B2 receptor was concentration dependent, and binding affinity was increased in the proprietary gas-enriched saline fluid of the instant disclosure compared to normal saline. Stabilization of Bradykinin binding to the B2 receptor is shown in Figure 75.

Figures 76-83 show data showing the ability of particular embodiments disclosed herein to affect regulatory T cells. The study involved irradiating antigen presenting cells, and introducing antigen and T cells.

Figure 84 shows that the inventive electrokinetically generated fluids decreased serum uptake of salmon calcitonin and an animal model. The results are consistent with enhancement of tight junctions.

Figures 85-89 show the expression levels of of tight junction-related proteins in lung tissue from the animal model used to generate the data of Figure 84.

Figures 90-94 show data obtained from human foreskin keratinocytes exposed to RDC1676-01 (sterile saline processed through the instant proprietary device with additional oxygen added; gas-enriched electrokinetically generated fluid (Rev) of the instant disclosure) showing up-regulation of NOS1 and 3, and Nostrin, NOS3.

Figures 95 and 96 show data supporting localized electrokinetic effects (voltage/current) occurring in a mixing device comprising insulated rotor and stator features to allow for detection of voltage/current effects during electrokinetic fluid generation.

Figures 97A-C show results of nuclear magnetic resonance (NMR) studies conducted to further characterize the fundamental nature of the inventive electrokinetically generated fluids. The electrokinetically generated fluids increased the ^{13}C -NMR line-widths of the reporter Trehalose solute.

Figures 98 and 99 show results of voltametric studies (i.e., square wave voltametry (Fig. 98) and stripping polarography (Fig. 99)) conducted to further characterize the fundamental nature of the inventive electrokinetically generated fluids. Square wave voltametry peak differences (with respect to control) unique to the electrokinetically generated fluids were observed at -0.14V, -0.47V, -1.02V and -1.36V. Pronounced polarographic peaks were seen at -0.9 volts for the electrokinetically generated Revera and Solas fluids, and the spectra of the non-electrokinetically generated blank and saline control fluids show characteristic peaks at -0.19 and -0.3 volts that are absent in the spectra for the electrokinetically generated fluids.

Figures 100-106 show results of patch clamping techniques that assessed the effects of the electrokinetically generated fluid test on epithelial cell membrane polarity and ion channel activity. The results indicate that the inventive electrokinetically generated fluids affect a voltage-dependent contribution of the whole-cell conductance.

Figures 107A-D and 108A-D show data indicating that the inventive electrokinetically generated fluids (e.g., RDC1676-00, RDC1676-01, RDC1676-02 and RDC1676-03) protected against methacholine-induced bronchoconstriction when administered alone or as diluents for albuterol sulfate in male guinea pigs.

Figures 109-114 show results of budesonide experiments performed to assess the airway anti-inflammatory properties of the inventive electrokinetically generated fluids in a Brown Norway rat ovalbumin sensitization model. The inventive electrokinetically generated fluids decreased eosinophil count, showed strong synergy with Budesonide in decreasing eosinophil count, decreased Penh values, increased Tidal Volume, decreased blood levels of Eotaxin, significantly enhanced the Blood levels of two major key anti-inflammatory cytokines, IL10 and Interferon gamma at 6 hours after challenge as a result of treatment with the inventive electrokinetically generated fluid (e.g., Rev 60) alone or in combination with Budesonide, and decreased systemic levels of Rantes. The data show that there is a substantial synergistic effect of Budesonide 750 ug/kg and the inventive electrokinetically generated fluids (e.g., Rev 60).

Figure 115 shows that the inventive electrokinetically generated fluid (e.g., Revera 60 and Solas) reduced DEP-induced TSLP receptor expression in bronchial epithelial cells (BEC) by approximately 90% and 50%, respectively, whereas normal saline (NS) had only a marginal effect.

Figure 116 shows the inventive electrokinetically generated fluid (e.g., Revera 60 and Solas) inhibited the DEP-induced cell surface bound MMP9 levels in bronchial epithelial cells by approximately 80%, and 70%, respectively, whereas normal saline (NS) had only a marginal effect.

DETAILED DESCRIPTION OF THE INVENTION

Certain embodiments disclosed herein provide novel compositions and methods for regulating or modulating intracellular signal transduction by modulation of at least one of cellular membranes, membrane potential and/or conductance, and membrane proteins such as membrane receptors, including but not limited to G protein coupled receptors. Particular aspects relate to modulating (e.g., treating or preventing) at least one symptom of a disease or condition associated with cellular membrane-mediated signal transduction (e.g., mediated by membrane receptors, and/or related to altered or aberrant function of membrane receptors), including G protein coupled receptors, in a

subject by administering a therapeutically effective amount of a composition comprising at least one electrokinetically generated fluid (including gas-enriched electrokinetically generated fluids) as disclosed herein.

Electrokinetically-generated fluids:

“Electrokinetically generated fluid,” as used herein, refers to Applicants’ inventive electrokinetically-generated fluids generated, for purposes of the working Examples herein, by the exemplary Mixing Device described in detail herein (see also US200802190088 and WO2008/052143, both incorporated herein by reference in their entirety). The electrokinetic fluids, as demonstrated by the data disclosed and presented herein, represent novel and fundamentally distinct fluids relative to prior art non-electrokinetic fluids, including relative to prior art oxygenated non-electrokinetic fluids (e.g., pressure pot oxygenated fluids and the like). As disclosed in various aspects herein, the electrokinetically-generated fluids have unique and novel physical and biological properties including, but not limited to the following:

In particular aspects, electrokinetically-generated fluids refers to fluids generated in the presence of hydrodynamically-induced, localized (e.g., non-uniform with respect to the overall fluid volume) electrokinetic effects (e.g., voltage/current pulses), such as device feature-localized effects as described herein. In particular aspects said hydrodynamically -induced, localized electrokinetic effects are in combination with surface-related double layer and/or streaming current effects as disclosed and discussed herein.

In particular aspects, the electrokinetically altered aqueous fluids are suitable to modulate ^{13}C -NMR line-widths of reporter solutes (e.g., Trehelose) dissolved therein. NMR line-width effects are in indirect method of measuring, for example, solute ‘tumbling’ in a test fluid as described herein in particular working Examples.

In particular aspects, the electrokinetically altered aqueous fluids are characterized by at least one of: distinctive square wave voltametry peak differences at any one of -0.14V, -0.47V, -1.02V and -1.36V; polarographic peaks at -0.9 volts; and an absence of polarographic peaks at -0.19 and -0.3 volts, which are unique to the electrokinetically generated fluids as disclosed herein in particular working Examples.

In particular aspects, the electrokinetically altered aqueous fluids are suitable to alter cellular membrane conductivity (e.g., a voltage-dependent contribution of the whole-cell conductance as measure in patch clamp studies disclosed herein).

In particular aspects, the electrokinetically altered aqueous fluids are oxygenated, wherein the oxygen in the fluid is present in an amount of at least 15, ppm, at least 25 ppm, at least 30 ppm, at least 40 ppm, at least 50 ppm, or at least 60 ppm dissolved oxygen at atmospheric pressure. In particular aspects, the electrokinetically altered aqueous fluids have less than 15 ppm, less than 10 ppm of dissolved oxygen at atmospheric pressure, or approximately ambient oxygen levels.

In particular aspects, the electrokinetically altered aqueous fluids are oxygenated, wherein the oxygen in the fluid is present in an amount between approximately 8 ppm and approximately 15 ppm, and in this case is sometimes referred to herein as "Solus."

In particular aspects, the electrokinetically altered aqueous fluid comprises at least one of solvated electrons (e.g., stabilized by molecular oxygen), and electrokinetically modified and/or charged oxygen species, and wherein in certain embodiments the solvated electrons and/or electrokinetically modified or charged oxygen species are present in an amount of at least 0.01 ppm, at least 0.1 ppm, at least 0.5 ppm, at least 1 ppm, at least 3 ppm, at least 5 ppm, at least 7 ppm, at least 10 ppm, at least 15 ppm, or at least 20 ppm.

In particular aspects, the electrokinetically altered aqueous fluids are suitable to alter cellular membrane structure or function (e.g., altering of a conformation, ligand binding activity, or a catalytic activity of a membrane associated protein) sufficient to provide for modulation of intracellular signal transduction, wherein in particular aspects, the membrane associated protein comprises at least one selected from the group consisting of receptors, transmembrane receptors (e.g., G-Protein Coupled Receptor (GPCR), TSLP receptor, beta 2 adrenergic receptor, bradykinin receptor, etc.), ion channel proteins, intracellular attachment proteins, cellular adhesion proteins, and integrins. In certain aspects, the effected G-Protein Coupled Receptor (GPCR) interacts with a G protein a subunit (e.g., G_{α_s} , G_{α_i} , G_{α_q} , and $G_{\alpha_{12}}$).

In particular aspects, the electrokinetically altered aqueous fluids are suitable to modulate intracellular signal transduction, comprising modulation of a calcium

dependant cellular messaging pathway or system (e.g., modulation of phospholipase C activity, or modulation of adenylate cyclase (AC) activity).

In particular aspects, the electrokinetically altered aqueous fluids are characterized by various biological activities (e.g., regulation of cytokines, receptors, enzymes and other proteins and intracellular signaling pathways) described in the working Examples and elsewhere herein.

In particular aspects, the electrokinetically altered aqueous fluids display synergy with Albuterol, and with Budesonide as shown in working Examples herein

In particular aspects, the electrokinetically altered aqueous fluids reduce DEP-induced TSLP receptor expression in bronchial epithelial cells (BEC) as shown in working Examples herein.

In particular aspects, the electrokinetically altered aqueous fluids inhibit the DEP-induced cell surface-bound MMP9 levels in bronchial epithelial cells (BEC) as shown in working Examples herein.

In particular aspects, the biological effects of the electrokinetically altered aqueous fluids are inhibited by diphtheria toxin, indicating that beta blockade, GPCR blockade and Ca channel blockade affects the activity of the electrokinetically altered aqueous fluids (e.g., on regulatory T cell function) as shown in working Examples herein.

In particular aspects, the physical and biological effects (e.g., the ability to alter cellular membrane structure or function sufficient to provide for modulation of intracellular signal transduction) of the electrokinetically altered aqueous fluids persists for at least two, at least three, at least four, at least five, at least 6 months, or longer periods, in a closed container (e.g., closed gas-tight container).

G Protein Receptor Associated Disorders and Conditions

Certain embodiments herein relate to therapeutic compositions and methods of treatment for a subject by preventing or alleviating at least one symptom of a G-Protein Receptor associated condition or disease. For example, the therapeutic compositions and/or methods disclosed herein may be useful for treating or preventing one or more condition or disease selected from the group consisting of inflammation, neurodegeneration, Alzheimer's Disease, aging, asthma, cystic fibrosis, developmental abnormalities of bone, hormone resistance (e.g. pseudohypoparathyroidism), hormone

hypersecretion (McCune-Albright syndrome caused by gain-of-function mutations), as well as retinal, endocrine, metabolic, and developmental disorders. In addition, the present therapeutic compositions and/or methods disclosed may be useful for preventing or ameliorating at least one symptom related to said conditions or diseases. Such symptoms may include altered bone growth, alterations in pigmentation of the skin, premature sexual development in children, psychological maladies, lung constriction (such as alveoli constriction, bronchial constriction, etc.), central nervous system disruption or degradation (including brain abnormalities), metabolic symptoms (such as insulin resistance), retinal disruption or degradation, and others.

In one particular embodiment, a gas-enriched fluid of the instant invention modulates or regulates GPCR expression, function, and/or regulation on airway smooth muscle cells (e.g., bronchial epithelial, pulmonary epithelial, etc.). In addition, the gas-enriched fluid disclosed herein may have a direct impact on airway smooth muscle growth or the secretion of various cytokines, chemokines, eicosanoids, or growth factors that orchestrate airway inflammation through actions on mesenchymal and/or infiltrating cells.

In one particular exemplary embodiment, the gas-enriched fluid of the instant invention modulates the binding affinity of Bradykinin for the Bradykinin B2 receptor. The B2 receptor is a G protein-coupled receptor that is associated with G_q and G_i . G_q stimulates phospholipase C to increase intracellular free calcium and G_i inhibits adenylate cyclase. The B2 receptor also stimulates the mitogen-activated protein kinase (MAPK) pathways, and is constitutively expressed in healthy tissues.

G_q dependent activation of protein kinase C and p42/p44 also promotes phosphorylation and activation of phospholipase A2 (PLA2), which contributes to rapid eicosanoid synthesis in airway smooth muscle cells stimulated with Bradykinin (acting on B2 Bradykinin receptors). Pang, and Knox, *Am. J. Physiol.* 273:L1132-L1140 (1997). Other effects reported to involve G_q activation by airway smooth muscle GPCRS include actin polymerization induced by lysophosphatidic acid, endothelin, or carbachol, which appears to occur via a Rho-dependent mechanism. Hirshman and Emala, *Am. J. Physiol.* 277:L653-L661 (1999).

While not wishing to be bound by any particular mechanism of action, upon activation of GPCRs, phospholipase C is activated, which promotes the hydrolysis of phosphoinositol 4,5-bisphosphate (PIP2) into the intracellular messengers 1,2-

diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). DAG remains membrane bound and promotes the translocation of protein kinase C from the cytoplasm to the membrane and its subsequent activation. Activated protein kinase C is capable of phosphorylating a number of substrates including calponin; protein kinase C-mediated phosphorylation of calponin results in a loss of calponin's ability to inhibit actomyosin ATPase. Winder and Walsh, *J. Biol. Chem.* 265:10148-10155 (1990). Protein kinase C also phosphorylates intermediates of MAPK signaling pathways, which activate various gene transcription factors involved in promoting cell growth, specifically of airway smooth muscle cells.

G_q-coupled receptors are also able to impact receptor tyrosine kinase-induced airway smooth muscle growth via a synergistic activation of p70S6K. Both protein kinase C and p42/p44 MAPK phosphorylate and stimulate the catalytic activity of phospholipase A2 (PLA2). Calcium binding to PLA2 triggers its association with the plasma or nuclear membrane and the subsequent cleaving and release of arachadonic acid (AA). The conversion of AA to prostaglandins and thromboxanes is facilitated by cyclo-oxygenase-2, a highly regulated enzyme upregulated by pro-inflammatory agents including lipopolysaccharide, cytokines and growth factors. The other product of PIP₂ hydrolysis, IP₃, translocates and binds to IP₃ receptors located on cellular calcium stores. Activation of IP₃ receptors results in the opening of calcium channels and calcium efflux into the cytosol. An increase in intracellular calcium results in calcium binding to calmodulin forming calcium-calmodulin complexes that further act on cellular proliferation and/or differentiation. Protein kinase C can also phosphorylate G_q coupled receptors, as well as phospholipase C and thereby inhibit G protein-coupled receptor-phospholipase C mediated phosphoinositide (PI) generation, and thus calcium flux. Protein kinase A phosphorylates the inositol 1,4,5-trisphosphate (IP₃) receptor to reduce its affinity for IP₃ and further limit calcium mobilization.

In certain diseases or conditions disclosed herein, altered or aberrant GPCR signaling results in aberrant cellular function and or gene expression. For example, airway smooth muscle contractile state and airway structure are the principal causes of increased airway resistance in asthma. Airway smooth muscle contractile state can be viewed as a function of: 1) the net sum of GPCR-mediated signals that result in establishing the level of the key contractile signaling molecule, calcium; and 2) the response of the cell's contractile machinery to calcium. Additionally, glucocorticoids

and beta-agonists may also regulate PGCR responsiveness, primarily via changes in receptor expression and coupling.

Inventive Gas-Enriched Fluids and Solutions

Diffusing or enriching a fluid with another fluid may result in a solution or suspension of the two fluids. In particular, enriching a liquid with a gas (e.g. oxygen) may be beneficial for certain applications, including therapeutic treatments. As utilized herein, "fluid," may generally refer to a liquid, a gas, a vapor, a mixture of liquids and/or gases, or any combination thereof, for any particular disclosed embodiment. Furthermore, in certain embodiments a "liquid" may generally refer to a pure liquid or may refer to a gel, sol, emulsion, fluid, colloid, dispersion, or mixture, as well as any combination thereof; any of which may vary in viscosity.

In particular embodiments disclosed herein, the dissolved gas comprises ambient air. In a preferred embodiment, the dissolved gas comprises oxygen. In another embodiment, the dissolved gas comprises nitric oxide.

There are several art-recognized methods of gas-enriching liquids (such as oxygen-enriching water). For example, a turbine aeration system can release air near a set of rotating blades of an impeller, which mixes the air or oxygen with the water, or water can be sprayed into the air to increase its oxygen content. Additionally, other systems on the market inject air or oxygen into the water and subject the water/gas to a large-scale vortex. Naturally occurring levels of oxygen in water are typically no more than 10 ppm (parts per million), which is considered to be a level of 100% dissolved oxygen. Tests on certain devices have shown that under ideal conditions, the device can attain upwards of approximately 20 ppm, or twice the natural oxygen levels of water. In certain embodiments, the oxygen level may be even higher.

Particular embodiments provided herein relate to a diffuser-processed therapeutic fluid as defined herein, comprising: a fluid host material; an infusion material diffused into the host material; and optionally, at least one therapeutic agent dispersed in the host material, wherein the infusion material comprises oxygen micro-bubbles in the host fluid, wherein the majority of the micro-bubbles are less than 0.2 microns, or preferably less than 0.1 microns in size. In certain embodiments, the dissolved oxygen level in the infused fluid host material may be maintained at greater than about 30 ppm at atmospheric pressure for at least 13 hours. In other particular

embodiments, the dissolved oxygen level in the infused fluid host material may be maintained at greater than 40 ppm at atmospheric pressure for at least 3 hours.

In additional embodiments, the infused fluid host material further comprises a saline solution. In further embodiments, the infused fluid host material maintains a dissolved oxygen level of at least about 20 ppm to about 40 ppm for a period of at least 100 days, preferably at least 365 days within a sealed container at atmospheric pressure. In certain embodiments, the infused fluid host material may have a dissolved oxygen level of at least 50 ppm at atmospheric pressure.

In certain embodiments, the infused fluid host material exhibits Rayleigh scattering for a laser beam shining therethrough for a selected period of time after the oxygen has been diffused into therein.

Table A illustrates various partial pressure measurements taken in a healing wound treated with an oxygen-enriched saline solution and in samples of the gas-enriched oxygen-enriched saline solution of the present invention.

TABLE A

TISSUE OXYGEN MEASUREMENTS	
Probe Z082BO	
In air: 171 mmHg	23° C
Column	Partial Pressure (mmHg)
B1	32-36
B2	169-200
B3	20-180*
B4	40-60
*wound depth minimal, majority >150, occasional 20 s	

BUBBLE SIZE MEASUREMENTS

Experimentation was performed to determine a size of the bubbles of gas diffused within the fluid by the mixing device 100. While experiments were not performed to measure directly the size of the bubbles, experiments were performed that established that the bubble size of the majority of the gas bubbles within the fluid was smaller than 0.1 microns. In other words, the experiments determined a size threshold value below which the sizes of the majority of bubbles fall.

This size threshold value or size limit was established by passing the output material 102 formed by processing a fluid and a gas in the mixing device 100 through a

0.22 micron filter and a 0.1 micron filter. In performing these tests, a volume of the first material 110, in this case, a fluid, and a volume of the second material 120, in this case, a gas, were passed through the mixing device 100 to generate a volume of the output material 102 (i.e., a fluid having a gas diffused therein). Sixty milliliters of the output material 102 was drained into a 60 ml syringe. The DO level of the fluid within the syringe was then measured using an Orion 862a. The Orion 862a is capable of measuring DO levels within a fluid. The fluid within the syringe was injected through a 0.22 micron filter into a 50 ml beaker. The filter comprised the Milipor Millex GP50 filter. The DO level of the material in the 50 ml beaker was then measured. The experiment was performed three times to achieve the results illustrated in Table II below.

Table II

DO IN SYRINGE	DO AFTER 0.22 MICRON FILTER
42.1 ppm	39.7 ppm
43.4 ppm	42.0 ppm
43.5 ppm	39.5 ppm

As can be seen, the DO levels measured within the syringe and the DO levels measured within the 50 ml beaker were not changed drastically by passing the output material 102 through the 0.22 micron filter. The implication of this experiment is that the bubbles of dissolved gas within the output material 102 are not larger than 0.22 microns otherwise there would be a significantly greater reduction in the DO levels in the output material 102 passed through the 0.22 micron filter.

A second test was performed in which the 0.1 micron filter was substituted for the 0.22 micron filter. In this experiment, saline solution was processed with oxygen in the mixing device 100 and a sample of the output material 102 was collected in an unfiltered state. The DO level of the unfiltered sample was 44.7 ppm. The output material 102 was filtered using the 0.1 micron filter and two additional samples were collected. The DO level of the first sample was 43.4 ppm. The DO level of the second sample was 41.4 ppm. Then, the filter was removed and a final sample was taken from the unfiltered output material 102. The final sample had a DO level of 45.4 ppm. These results were consistent with those seen using the Millipore 0.2 micron filter. These results lead to the conclusion that there is a trivial reduction in the DO levels of

the output material 102 passed through the 0.1 micron filter providing an indication that the majority of the bubbles in the processed saline solution are no greater than 0.1 micron in size. The DO level test results described above were achieved using Winkler Titration.

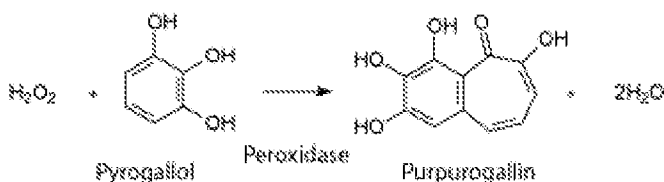
As appreciated in the art, the double-layer (interfacial) (DL) appears on the surface of an object when it is placed into a liquid. This object, for example, might be that of a solid surface (e.g., rotor and stator surfaces), solid particles, gas bubbles, liquid droplets, or porous body. In the mixing device 100, bubble surfaces represent a significant portion of the total surface area present within the mixing chamber that may be available for electrokinetic double-layer effects. Therefore, in addition to the surface area and retention time aspects discussed elsewhere herein, the relatively small bubble sizes generated within the mixer 100 compared to prior art devices 10, may also contribute, at least to some extent, to the overall electrokinetic effects and output fluid properties disclosed herein. Specifically, in preferred embodiments, as illustrated by the mixer 100, all of the gas is being introduced via apertures on the rotor (no gas is being introduced through stator apertures. Because the rotor is rotating at a high rate (e.g., 3,400 rpm) generating substantial shear forces at and near the rotor surface, the bubble size of bubbles introduced via, and adjacent to the spinning rotor surface apertures would be expected to be substantially (e.g., 2 to 3-times smaller) smaller than those introduced via and near the stationary stator. The average bubble size of the prior art device 10 may, therefore, be substantially larger because at least half of the gas is introduced into the mixing chamber from the stationary stator apertures. Because the surface area of a sphere surface varies with r^2 , any such bubble component of the electrokinetic surface area of the mixing device 100 may be substantially greater than that of the prior art diffusion device 10.

Compositions comprising hydrated (solvated) electrons imparted to the inventive compositions by the inventive processes

In certain embodiments as described herein (see under "Double-layer"), the gas-enriched fluid is generated by the disclosed electromechanical processes in which molecular oxygen is diffused or mixed into the fluid and may operate to stabilize charges (e.g., hydrated (solvated) electrons) imparted to the fluid. Without being bound by theory or mechanism, certain embodiments of the present invention relate to a oxygen-enriched fluid (output material) comprising charges (e.g., hydrated (solvated)

electrons) that are added to the materials as the first material is mixed with oxygen in the inventive mixer device to provide the combined output material. According to particular aspects, these hydrated (solvated) electrons (alternately referred to herein as 'solvated electrons') are stabilized in the inventive solutions as evidenced by the persistence of assayable effects mediated by these hydrated (solvated) electrons. Certain embodiments may relate to hydrated (solvated) electrons and/or water-electron structures, clusters, etc., (See, for example, Lee and Lee, *Bull. Kor. Chem. Soc.* 2003, v. 24, 6; 802-804; 2003).

Horseradish peroxidase (HRP) effects. Horseradish peroxidase (HRP) is isolated from horseradish roots (*Amoracia rusticana*) and belongs to the ferroporphyrin group (Heme group) of peroxidases. HRP readily combines with hydrogen peroxide or other hydrogen donors to oxidize the pyrogallol substrate. Additionally, as recognized in the art, HRP facilitates auto-oxidative degradation of indole-3-acetic acid in the absence of hydrogen peroxide (see, e.g., *Heme Peroxidases*, H. Brian Dunford, Wiley-VCH, 1999, Chapter 6, pages 112-123, describing that auto-oxidation involves a highly efficient branched-chain mechanism; incorporated herein by reference in its entirety). The HRP reaction can be measured in enzymatic activity units, in which Specific activity is expressed in terms of pyrogallol units. One pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C. This purpurogallin (20 sec) unit is equivalent to approx. 18 μ M units per min at 25°C.



It is known that Horseradish peroxidase enzyme catalyzes the auto-oxidation of pyrogallol by way of facilitating reaction with the molecular oxygen in a fluid. (Khajehpour et al., *PROTEINS: Struct, Funct, Genet.* 53: 656-666 (2003)). It is also known that oxygen binds the *heme* pocket of horseradish peroxidase enzyme through a hydrophobic pore region of the enzyme (between Phe68 and Phe142), whose conformation likely determines the accessibility of oxygen to the interior. According to particular aspects, and without being bound by mechanism, because surface charges on proteins are known in the protein art to influence protein structure, the solvated

electrons present in the inventive gas-enriched fluid may act to alter the conformation of the horseradish peroxidase such that greater oxygen accessibility may result. The greater accessibility of oxygen to the prosthetic heme pocket of the horseradish peroxidase enzyme may in turn allow for increased HRP reactivity, when compared with prior art oxygenated fluids (pressure-pot, fine-bubbled).

In any event, according to particular aspects, production of output material using the inventive methods and devices comprises a process involving: an interfacial double layer that provides a charge gradient; movement of the materials relative to surfaces pulling charge (e.g., electrons) away from the surface by virtue of a triboelectric effect, wherein the flow of material produces a flow of solvated electrons. Moreover, according to additional aspects, and without being bound by mechanism, the orbital structure of diatomic oxygen creates charge imbalances (e.g., the two unpaired electrons affecting the hydrogen bonding of the water) in the hydrogen bonding arrangement within the fluid material (water), wherein electrons are solvated and stabilized within the imbalances.

Several chemical tests of the inventive oxygen-enriched fluid for the presence of hydrogen peroxide were conducted as described below, and none of these tests were positive (sensitivity of 0.1 ppm hydrogen peroxide). Thus, the inventive oxygen-enriched fluid of the instant application contain no, or less than 0.1 ppm hydrogen peroxide.

According to particular aspects, despite the absence of hydrogen peroxide, the inventive combination of oxygen-enrichment and solvated electrons imparted by the double-layer effects and configuration of the presently claimed devices may act to alter the conformation and/or heme group accessibility of the horseradish peroxidase.

Glutathione Peroxidase Study

The inventive oxygen-enriched output fluid material was tested for the presence of hydrogen peroxide by testing the reactivity with glutathione peroxidase using a standard assay (Sigma). Briefly, glutathione peroxidase enzyme cocktail was constituted in deionized water and the appropriate buffers. Water samples were tested by adding the enzyme cocktail and inverting. Continuous spectrophotometric rate determination was made at A_{340} nm, and room temperature (25 degrees Celsius). Samples tested were: 1. deionized water (negative control), 2. inventive oxygen-enriched fluid at low concentration, 3. inventive oxygen-enriched fluid at high

concentration, 4. hydrogen peroxide (positive control). The hydrogen peroxide positive control showed a strong reactivity, while none of the other fluids tested reacted with the glutathione.

Device for Generating Gas-Enriched Fluids or Solutions

Description of the Related Art

Figure 1 provides a partial block diagram, partial cross-sectional view of a prior art device 10 for diffusing or emulsifying one or two gaseous or liquid materials ("infusion materials") into another gaseous or liquid material ("host material") reproduced from U.S. Patent No. 6,386,751, incorporated herein by reference in its entirety. The device 10 includes a housing configured to house a stator 30 and a rotor 12. The stator 30 encompasses the rotor 12. A tubular channel 32 is defined between the rotor 12 and the stator 30. The generally cylindrically shaped rotor 12 has a diameter of about 7.500 inches and a length of about 6.000 inches providing a length to diameter ratio of about 0.8.

The rotor 12 includes a hollow cylinder, generally closed at both ends. A gap exists between each of the first and second ends of the rotor 12 and a portion of the housing 34. A rotating shaft 14 driven by a motor 18 is coupled to the second end of the rotor 12. The first end of the rotor 12 is coupled to an inlet 16. A first infusion material passes through the inlet 16 and into the interior of the rotor 12. The first infusion material passes from the interior of the rotor 12 and into the channel 32 through a plurality of openings 22 formed in the rotor 12.

The stator 30 also has openings 22 formed about its circumference. An inlet 36 passes a second infusion material to an area 35 between the stator 30 and the housing 34. The second infusion material passes out of the area 35 and into the channel 32 through openings 22.

An external pump (not shown) is used to pump the host material into a single inlet port 37. The host material passes through a single inlet port 37 and into the channel 32 where it encounters the first and second infusion materials, which enter the channel 32 through openings 22. The infusion materials may be pressurized at their source to prevent the host material from passing through openings 22.

The inlet port 37, is configured and positioned such that it is located along only a relatively small portion (< about 5%) of the annular inlet channel 32, and is substantially

parallel to the axis of rotation of the rotor 12 to impart an axial flow toward a portion of the channel 32 into the host material.

Unfortunately, before entering the tubular channel 32, the host material must travel in tortuous directions other than that of the axial flow (e.g., including in directions substantially orthogonal thereto) and down into and between the gap formed between the first end of the rotor 12 and the housing 34 (i.e., down a portion of the first end of the rotor adjacent to the inlet 16 between the end of the rotor 12 and the housing 34). The non-axial and orthogonal flow, and the presence of the host material in the gap between the first end of the rotor 12 and the housing 34 causes undesirable and unnecessary friction. Further, it is possible for a portion of the host material to become trapped in eddy currents swirling between the first end of the rotor and the housing. Additionally, in the device 10, the host material must negotiate at least two right angles to enter any aspect of the annular inlet of the tubular channel 32.

A single outlet port 40 is formed in the housing 34. The combined host material and infusion material(s) exit the channel 32 via the outlet 40. The outlet port 40, which is also located along only a limited portion (< about 5%) of the annular outlet of tubular channel 32, is substantially parallel to the axis of rotation of the rotor 12 to impart or allow for an axial flow of the combined materials away from the limited portion of the annular outlet of tubular channel 32 into the outlet port 40. An external pump 42 is used to pump the exiting fluid through the outlet port 40.

Unfortunately, before exiting the channel 32, a substantial portion of the exiting material must travel in a tortuous direction other than that of the axial flow (e.g., including in directions substantially orthogonal thereto) and down into and between the gap formed between the second end of the rotor 12 and the housing 34 (i.e., down a portion of the second end of the rotor adjacent to the shaft 14 between the end of the rotor 12 and the housing 34). As mentioned above, the non-axial and orthogonal flow, and the presence of the host material in the other gap between the end (in this case, the second end) of the rotor 12 and the housing 34 causes additional undesirable and unnecessary friction. Further, it is possible for a portion of the host material to become trapped in eddy currents swirling between the second end of the rotor and the housing. Additionally, in the device 10, a substantial portion of the exiting combined material must negotiate at least two right angles as it exits from the annular exit of the tubular channel 32 into the outlet port 40.

As is apparent to those of ordinary skill in the art, the inlet port 37 imparts only an axial flow to the host material. Only the rotor 21 imparts a circumferential flow into the host material. Further, the outlet port 40 imparts or provides for only an axial flow into the exiting material. Additionally, the circumferential flow velocity vector is imparted to the material only after it enters the annular inlet 37 of the tubular channel 32, and subsequently the circumferential flow vector must be degraded or eliminated as the material enters the exit port 40. There is, therefore, a need for a progressive circumferential acceleration of the material as it passes in the axial direction through the channel 32, and a circumferential deceleration upon exit of the material from the channel 32. These aspects, in combination with the tortuous path that the material takes from the inlet port 37 to the outlet port 40, create a substantial friction and flow resistance over the path that is accompanied by a substantial pressure differential (26 psi, at 60 gallons/min flow rate) between the inlet 37 and outlet 40 ports, and these factors, *inter alia*, combine to reduce the overall efficiency of the system.

Electrokinetically Oxygen-Enriched Aqueous Fluids and Solutions

Figure 2 provides a block diagram illustrating some of the components of a mixing device 100 and the flow of material into, within, and out of the device. The mixing device 100 combines two or more input materials to form an output material 102, which may be received therefrom into a storage vessel 104. The mixing device 100 agitates the two or more input materials in a novel manner to produce an output material 102 having novel characteristics. The output material 102 may include not only a suspension of at least one of the input materials in at least one of the other input materials (e.g., emulsions) but also a novel combination (e.g., electrostatic combinations) of the input materials, a chemical compound resulting from chemical reactions between the input materials, combinations having novel electrostatic characteristics, and combinations thereof.

The input materials may include a first material 110 provided by a source 112 of the first material, a second material 120 provided by a source 122 of the second material, and optionally a third material 130 provided by a source 132 of the third material. The first material 110 may include a liquid, such as water, saline solution, chemical suspensions, polar liquids, non-polar liquids, colloidal suspensions, cell growing media, and the like. In some embodiments, the first material 110 may include the output material 102 cycled back into the mixing device 100. The second

material 120 may consist of or include a gas, such as oxygen, nitrogen, carbon dioxide, carbon monoxide, ozone, sulfur gas, nitrous oxide, nitric oxide, argon, helium, bromine, and combinations thereof, and the like. In preferred embodiments, the gas is or comprises oxygen. The optional third material 130 may include either a liquid or a gas. In some embodiments, the third material 130 may be or include the output material 102 cycled back into the mixing device 100 (e.g., to one or more of the pumps 210, 220 or 230, and/or into the chamber 310, and/or 330).

Optionally, the first material 110, the second material 120, and the optional third material 130 may be pumped into the mixing device 100 by an external pump 210, an external pump 220, and an external pump 230, respectively. Alternatively, one or more of the first material 110, the second material 120, and the optional third material 130 may be stored under pressure in the source 112, the source 122, and the source 132, respectively, and may be forced into the mixing device 100 by the pressure. The invention is not limited by the method used to transfer the first material 110, the second material 120, and optionally, the third material 130 into the mixing device 100 from the source 112, the source 122, and the source 132, respectively.

The mixing device 100 includes a first chamber 310 and a second chamber 320 flanking a mixing chamber 330. The three chambers 310, 320, and 330 are interconnected and form a continuous volume.

The first material 110 is transferred into the first chamber 310 and flows therefrom into the mixing chamber 330. The first material 110 in the first chamber 310 may be pumped into the first chamber 310 by an internal pump 410. The second material 120 is transferred into the mixing chamber 330. Optionally, the third material 130 may be transferred into the mixing chamber 330. The materials in the mixing chamber 330 are mixed therein to form the output material 102. Then, the output material 102 flows into the second chamber 320 from which the output material 102 exits the mixing device 100. The output material 102 in the mixing chamber 330 may be pumped into the second chamber 320 by an internal pump 420. Optionally, the output material 102 in the second chamber 320 may be pumped therefrom into the storage vessel 104 by an external pump 430 (e.g., alone or in combination with the internal pump 410 and/or 420).

In particular aspects, a common drive shaft 500 powers both the internal pump 410 and the internal pump 420. The drive shaft 500 passes through the mixing chamber 330 and provides rotational force therein that is used to mix the first

material 110, the second material 120, and optionally, the third material 130 together. The drive shaft 500 is powered by a motor 510 coupled thereto.

Figure 3 provides a system 512 for supplying the first material 110 to the mixing device 100 and removing the output material 102 from the mixing device 100. In the system 512, the storage vessel 104 of the output material 102 and the source 112 of the first material 110 are combined. The external pump 210 is coupled to the combined storage vessel 104 and source 112 by a fluid conduit 514 such as hose, pipe, and the like. The external pump 210 pumps the combined first material 110 and output material 102 from the combined storage vessel 104 and source 112 through the fluid conduit 514 and into a fluid conduit 516 connecting the external pump 210 to the mixing device 100. The output material 102 exits the mixing device 100 through a fluid conduit 518. The fluid conduit 518 is coupled to the combined storage vessel 104 and source 112 and transports the output material 102 exiting the mixing device 100 to the combined storage vessel 104 and source 112. The fluid conduit 518 includes a valve 519 that establishes an operating pressure or back pressure within the mixing device 100.

Referring to Figures 2, 4-9, and 11, a more detailed description of various components of an embodiment of the mixing device 100 will be provided. The mixing device 100 is scalable. Therefore, dimensions provided with respect to various components may be used to construct an embodiment of the device or may be scaled to construct a mixing device of a selected size.

Turning to Figure 4, the mixing device 100 includes a housing 520 that houses each of the first chamber 310, the mixing chamber 330, and the second chamber 320. As mentioned above, the mixing device 100 includes the drive shaft 500, which rotates during operation of the device. Therefore, the mixing device 100 may vibrate or otherwise move. Optionally, the mixing device 100 may be coupled to a base 106, which may be affixed to a surface such as the floor to maintain the mixing device 100 in a substantially stationary position.

The housing 520 may be assembled from two or more housing sections. By way of example, the housing 520 may include a central section 522 flanked by a first mechanical seal housing 524 and a second mechanical seal housing 526. A bearing housing 530 may be coupled to the first mechanical seal housing 524 opposite the central section 522. A bearing housing 532 may be coupled to the second mechanical

seal housing 526 opposite the central section 522. Optionally, a housing section 550 may be coupled to the bearing housings 530.

Each of the bearing housings 530 and 532 may house a bearing assembly 540 (see Figures 5 and 6). The bearing assembly 540 may include any suitable bearing assembly known in the art including a model number "202SZZST" manufactured by SKF USA Inc, of Kulpville, Pennsylvania, operating a website at www.skf.com.

Seals may be provided between adjacent housing sections. For example, o-ring 560 (see Figure 5) may be disposed between the housing section 550 and the bearing housing 530, o-ring 562 (see Figure 5) may be disposed between the first mechanical seal housing 524 and the central section 522, and o-ring 564 (see Figure 6) may be disposed between the second mechanical seal housing 526 and the central section 522.

MIXING CHAMBER 330

Turning now to Figure 7, the mixing chamber 330 is disposed inside the central section 522 of the housing 520 between the first mechanical seal housing 524 and the second mechanical seal housing 526. The mixing chamber 330 is formed between two components of the mixing device 100, a rotor 600 and a stator 700. The rotor 600 may have a sidewall 604 with an inside surface 605 defining a generally hollow inside portion 610 and an outside surface 606. The sidewall 604 may be about 0.20 inches to about 0.75 inches thick. In some embodiments, the sidewall 604 is about 0.25 inches thick. However, because the mixing device 100 may be scaled to suit a particular application, embodiments of the device having a sidewall 604 that is thicker or thinner than the values provided are within the scope of the present teachings. The sidewall 604 includes a first end portion 612 and a second end portion 614 and a plurality of through-holes 608 formed between the first end portion 612 and the second end portion 614. Optionally, the outside surface 606 of the sidewall 604 may include other features such as apertures, projections, textures, and the like. The first end portion 612 has a relieved portion 616 configured to receive a collar 618 and the second end portion 614 has a relieved portion 620 configured to receive a collar 622.

The rotor 600 is disposed inside the stator 700. The stator 700 has a sidewall 704 with an inside surface 705 defining a generally hollow inside portion 710 into which the rotor 600 is disposed. The sidewall 704 may be about 0.1 inches to about 0.3 inches thick. In some embodiments, the sidewall 604 is about 1.5 inches

thick. The stator 700 may be non-rotatably coupled to the housing 520 in a substantially stationary position. Alternatively, the stator 700 may be integrally formed with the housing 520. The sidewall 704 has a first end portion 712 and a second end portion 714. Optionally, a plurality of apertures 708 are formed in the sidewall 704 of the stator 700 between the first end portion 712 and the second end portion 714. Optionally, the inside surface 705 of the sidewall 704 may include other features such as through-holes, projections, textures, and the like.

The rotor 600 rotates with respect to the stationary stator 700 about an axis of rotation "a" in a direction indicated by arrow "C3" in Figure 9. Each of the rotor 600 and the stator 700 may be generally cylindrical in shape and have a longitudinal axis. The rotor 600 has an outer diameter "D1" and the stator 700 may have an inner diameter "D2." The diameter "D1" may range, for example, from about 0.5 inches to about 24 inches. In some embodiments, the diameter "D1" is about 3.04 inches. In some embodiments, the diameter "D1" is about 1.7 inches. The diameter "D2," which is larger than the diameter "D1," may range from about 0.56 inches to about 24.25 inches. In some embodiments, the diameter "D2" is about 4 inches. Therefore, the mixing chamber 330 may have a ring-shaped cross-sectional shape that is about 0.02 inches to about 0.125 inches thick (i.e., the difference between the diameter "D2" and the diameter "D1"). In particular embodiments, the mixing chamber 330 is about 0.025 inches thick. The channel 32 between the rotor 12 and the stator 34 of prior art device 10 (see Figure 1) has a ring-shaped cross-sectional shape that is about 0.09 inches thick. Therefore, in particular embodiments, the thickness of the mixing chamber 330 is less than about one third of the channel 32 of the prior art device 10.

The longitudinal axis of the rotor 600 may be aligned with its axis of rotation "a." The longitudinal axis of the rotor 600 may be aligned with the longitudinal axis of the stator 700. The rotor 600 may have a length of about 3 inches to about 6 inches along the axis of rotation "a." In some embodiments, the rotor 600 may have a length of about 5 inches along the axis of rotation "a." The stator 700 may have a length of about 3 inches to about 6 inches along the axis of rotation "a." In some embodiments, the stator 700 may have a length of about 5 inches along the axis of rotation "a."

While the rotor 600 and the stator 700 have been depicted as having a generally cylindrical shape, those of ordinary skill in the art appreciate that alternate shapes may be used. For example, the rotor 600 and the stator 700 may be conically, spherically, arbitrarily shaped, and the like. Further, the rotor 600 and the stator 700 need not be

identically shaped. For example, the rotor 600 may be cylindrically shaped and the stator 700 rectangular shaped or vice versa.

The apertures 708 of the stator 700 and the through-holes 608 depicted in Figures 4—7 are generally cylindrically shaped. The diameter of the through-holes 608 may range from about 0.1 inches to about 0.625 inches. The diameter of the apertures 708 may range from about 0.1 inches to about 0.625 inches. One or more of apertures 708 of the stator 700 may have a diameter that differs from the diameters of the other apertures 708. For example, the apertures 708 may increase in diameter from the first end portion 712 of the stator 700 to the second end portion 714 of the stator 700, the apertures 708 may decrease in diameter from the first end portion 712 of the stator 700 to the second end portion 714 of the stator 700, or the diameters of the apertures 708 may vary in another manner along the stator 700. One or more of through-holes 608 of the rotor 600 may have a diameter that differs from the diameters of the other through-holes 608. For example, the through-holes 608 may increase in diameter from the first end portion 612 of the rotor 600 to the second end portion 614 of the rotor 600, the through-holes 608 may decrease in diameter from the first end portion 612 of the rotor 600 to the second end portion 614 of the rotor 600, or the diameters of the through-holes 608 may vary in another manner along the rotor 600.

As described below with reference to alternate embodiments, the apertures 708 and the through-holes 608 may have shapes other than generally cylindrical and such embodiments are within the scope of the present invention. For example, the through-holes 608 may include a narrower portion, an arcuate portion, a tapered portion, and the like. Referring to Figures 7, each of the through-holes 608 includes an outer portion 608A, a narrow portion 608B, and a tapered portion 608C providing a transition between the outer portion 608A and the narrow portion 608B. Similarly, the apertures 708 may include a narrower portion, an arcuate portion, a tapered portion, and the like.

Figure 8 provides a non-limiting example of a suitable arrangement of the apertures 708 of the stator 700 and the through-holes 608 of the rotor 600. The apertures 708 of the stator 700 may be arranged in substantially parallel lateral rows “SLAT-1” through “SLAT-6” substantially orthogonal to the axis of rotation “a.” The apertures 708 of the stator 700 may also be arranged in substantially parallel longitudinal rows “SLONG-1” through “SLONG-7” substantially parallel with the axis of rotation “a.” In other words, the apertures 708 of the stator 700 may be arranged in a

grid-like pattern of orthogonal rows (i.e., the lateral rows are orthogonal to the longitudinal rows) having the longitudinal rows "SLONG-1" through "SLONG-7" substantially parallel with the axis of rotation "a."

Like the apertures 708 of the stator 700, the through-holes 608 of the rotor 600 may be arranged in substantially parallel lateral rows "RLAT-1" through "RLAT-6" substantially orthogonal to the axis of rotation "a." However, instead of being arranged in a grid-like pattern of orthogonal rows, the through-holes 608 of the rotor 600 may also be arranged in substantially parallel rows "RLONG-1" through "RLONG-7" that extend longitudinally along a helically path. Alternatively, the through-holes 608 of the rotor 600 may also be arranged in substantially parallel rows "RLONG-1" through "RLONG-7" that extend longitudinally at an angle other than parallel with the axis of rotation "a."

The apertures 708 of the stator 700 and the through-holes 608 of the rotor 600 may be configured so that when the rotor 600 is disposed inside the stator 700 the lateral rows "SLAT-1" to "SLAT-6" at least partially align with the lateral rows "RLAT-1" to "RLAT-6," respectively. In this manner, as the rotor 600 rotates inside the stator 700, the through-holes 608 pass by the apertures 708.

The through-holes 608 in each of the lateral rows "RLAT-1" to "RLAT-6" may be spaced apart laterally such that all of the through-holes 608 in the lateral row align, at least partially, with the apertures 708 in a corresponding one of the lateral rows "SLAT-1" to "SLAT-6" of the stator 700 at the same time. The longitudinally extending rows "RLONG-1" through "RLONG-6" may be configured such that the through-holes 608 in the first lateral row "RLAT-1" in each of the longitudinally extending rows passes completely by the apertures 708 of the corresponding lateral row "SLAT-1" before the through-holes 608 in the last lateral row "RLAT-6" begin to partially align with the apertures 708 of the corresponding last lateral row "SLAT-6" of the stator 700.

While, in Figure 8, six lateral rows and six longitudinally extending rows have been illustrated with respect to the rotor 600 and six lateral rows and seven longitudinally extending rows have been illustrated with respect stator 700, it is apparent to those of ordinary skill in the art that alternate numbers of lateral rows and/or longitudinal rows may be used with respect to the rotor 600 and/or stator 700 without departing from the present teachings.

To ensure that only one pair of openings between corresponding lateral rows will be coincident at any one time, the number of apertures 708 in each of the lateral

rows "SLAT-1" to "SLAT-6" on the stator 700 may differ by a predetermined number (e.g., one, two, and the like) the number of through-holes 608 in each of the corresponding lateral rows "RLAT-1" to "RLAT-6" on the rotor 600. Thus, for example, if lateral row "RLAT-1" has twenty through-holes 608 evenly spaced around the circumference of rotor 600, the lateral row "SLAT-1" may have twenty apertures 708 evenly spaced around the circumference of stator 700.

Returning to Figure 7, the mixing chamber 330 has an open first end portion 332 and an open second end portion 334. The through-holes 608 formed in the sidewall 604 of the rotor 600 connect the inside portion 610 of the rotor 600 with the mixing chamber 330.

The rotor 600 is rotated inside the stator 700 by the drive shaft 500 aligned with the axis of rotation "a" of the rotor 600. The drive shaft 500 may be coupled to the first end portion 612 and the second end portion 614 of the rotor 600 and extend through its hollow inside portion 610. In other words, a portion 720 of the drive shaft 500 is disposed in the hollow inside portion 610 of the rotor 600.

The collar 618 is configured to receive a portion 721 of the drive shaft 500 disposed in the hollow inside portion 610 and the collar 622 is configured to receive a portion 722 of the drive shaft 500 disposed in the hollow inside portion 610.

The portion 721 has an outer diameter "D3" that may range from about 0.5 inches to about 2.5 inches. In some embodiments, the diameter "D3" is about 0.625 inches. The portion 722 has an outer diameter "D4" that may be substantially similar to the diameter "D3," although, this is not required. The diameter "D4" may range from about 0.375 inches to about 2.5 inches.

The rotor 600 may be non-rotationally affixed to the portion 721 and the portion 722 of the drive shaft 500 by the collar 618 and the collar 622, respectively. By way of example, each of the collars 618 and 622 may be installed inside relieved portions 616 and 620, respectively. Then, the combined rotor 600 and collars 618 and 622 may be heated to expand them. Next, the drive shaft 500 is inserted through the collars 618 and 622 and the assembly is allowed to cool. As the collars 618 and 622 shrink during cooling, they tighten around the portions 722A and 722B of the drive shaft 500, respectively, gripping it sufficiently tightly to prevent the drive shaft 500 from rotating relative to the rotor 600. The collar 618, which does not rotate with respect to either the portion 721 or the relieved portion 616, translates the rotation of the drive shaft 500 to the first end portion 612 of the rotor 600. The collar 622, which does

not rotate with respect to either the portion 722 or the relieved portion 620, translates the rotation of the drive shaft 500 to the second end portion 614 of the rotor 600. The drive shaft 500 and the rotor 600 rotate together as a single unit.

The drive shaft 500 may have a first end portion 724 (see Figure 5) and a second end portion 726 (see Figure 6). The first end portion 724 may have a diameter "D5" of about 0.5 inches to about 1.75 inches. In particular embodiments, the diameter "D5" may be about 1.25 inches. The second end portion 726 may have a diameter "D6" that may be substantially similar to diameter "D5."

The second material 120 may be transported into the mixing chamber 330 through one of the first end portion 724 and the second end portion 726 of the rotating drive shaft 500. The other of the first end portion 724 and the second end portion 726 of the drive shaft 500 may be coupled to the motor 510. In the embodiment depicted in Figures 5 and 6, the second material 120 is transported into the mixing chamber 330 through the first end portion 724 and the second end portion 726 of the drive shaft 500 is coupled to the motor 510.

Turning to Figure 5, the drive shaft 500 may have a channel 728 formed therein that extends from first end portion 724 into the portion 720 disposed in the inside portion 610 of the rotor 600. The channel 728 has an opening 730 formed in the first end portion 724. When the mixing device 100 is operating, the second material 120 is introduced into the channel 728 through the opening 730.

A valve 732 may be disposed inside a portion of the channel 728 located in the first end portion 724 of the drive shaft 500. The valve 732 may restrict or otherwise control the backward flow of the second material 120 from inside the hollow inside portion 610 through the channel 728 and/or the forward flow of the second material 120 into the channel 728. The valve 732 may include any valve known in the art including a check valve. A suitable check valve includes a part number "CKFA1876205A," free flow forward check valve, manufactured by The Lee Company USA having an office in Bothell, WA and operating a website at www.theleeco.com.

The drive shaft 500 may include an aperture 740 located in the inside portion 610 of the rotor 600 that connects the channel 728 with the inside portion 610 of the rotor 600. While only a single aperture 740 is illustrated in Figure 5, it is apparent to those of ordinary skill in the art that multiple apertures may be used to connect the channel 728 with the inside portion 610 of the rotor 600.

Referring to Figure 2, optionally, the external pump 220 may pump the second material 120 into the mixing device 100. The pump 220 may include any suitable pump known in the art. By way of non-limiting example, the pump 220 may include any suitable pump known in the art including a diaphragm pump, a chemical pump, a peristaltic pump, a gravity fed pump, a piston pump, a gear pump, a combination of any of the aforementioned pumps, and the like. If the second material 120 is a gas, the gas may be pressurized and forced into the opening 730 formed in the first end portion 724 of the drive shaft 500 by releasing the gas from the source 122.

The pump 220 or the source 122 is coupled to the channel 728 by the valve 732. The second material 120 transported inside the channel 728 exits the channel 728 into the inside portion 610 of the rotor 600 through the aperture 740. The second material 120 subsequently exits the inside portion 610 of the rotor 600 through the through-holes 608 formed in the sidewall 608 of the rotor 600.

Referring to Figure 5, the mixing device 100 may include a seal assembly 750 coupled to the first end portion 724 of the drive shaft 500. The seal assembly 750 is maintained within a chamber 752 defined in the housing 520. The chamber 752 has a first end portion 754 spaced across the chamber from a second end portion 756. The chamber 752 also includes an input port 758 and an output port 759 that provide access into the chamber 752. The chamber 752 may be defined by housing section 550 and the bearing housing 530. The first end portion 754 may be formed in the housing section 550 and the second end portion 756 may be adjacent to the bearing housing 530. The input port 758 may be formed in the bearing housing 530 and the output port 759 may be formed in the housing section 550.

The seal assembly 750 includes a first stationary seal 760 installed in the first end portion 754 of the chamber 752 in the housing section 550 and the bearing housing 530. The first stationary seal 760 extends around a portion 762 of the first end portion 724 of the drive shaft 500. The seal assembly 750 also includes a second stationary seal 766 installed in the second end portion 756 of the chamber 752 in the bearing housing 530. The second stationary seal 766 extends around a portion 768 of the first end portion 724 of the drive shaft 500.

The seal assembly 750 includes a rotating assembly 770 that is non-rotatably coupled to the first end portion 724 of the drive shaft 500 between the portion 762 and the portion 768. The rotating assembly 770 rotates therewith as a unit. The rotating assembly 770 includes a first seal 772 opposite a second seal 774. A biasing

member 776 (e.g., a spring) is located between the first seal 772 and the second seal 774. The biasing member 776 biases the first seal 772 against the first stationary seal 760 and biases the second seal 774 against the second stationary seal 766.

A cooling lubricant is supplied to the chamber 752 and around rotating assembly 770. The lubricant enters the chamber 752 through the input port 758 and exits the chamber 752 through output port 759. The lubricant may lubricate the bearing assembly 540 housed by the bearing housing 530. A chamber 570 may be disposed between the bearing housing 530 and the mechanical seal housing 524. The bearing housing 530 may also include a second input port 759 connected to the chamber 570 into which lubricant may be pumped. Lubricant pumped into the chamber 570 may lubricate the bearing assembly 540. The seal assembly 750 may significantly, if not greatly, reduce frictional forces within this portion of the device caused by the rotation of the rotor 600 and may increase the active life of the seals 770. The seals may include surfaces constructed using silicon carbide.

Referring to Figure 9, as the rotor 600 rotates about the axis of rotation "a" in the direction indicated by arrow "C1," the rotor expels the second material 120 into the mixing chamber 330. The expelled bubbles, droplets, particles, and the like of the second material 120 exit the rotor 600 and are imparted with a circumferential velocity (in a direction indicated by arrow "C3") by the rotor 600. The second material 120 may be forced from the mixing chamber 330 by the pump 220 (see Figure 2), the centrifugal force of the rotating rotor 600, buoyancy of the second material 120 relative to the first material 110, and a combination thereof.

MOTOR 510

Returning to Figure 6, the second end portion 726 of the drive shaft 500 may be coupled to a rotating spindle 780 of a motor 510 by a coupler 900. The spindle 780 may have a generally circular cross-sectional shape with a diameter "D7" of about 0.25 inches to about 2.5 inches. In particular embodiments, the diameter "D7" may be about 0.25 inches to about 1.5 inches. While in the embodiment depicted in Figure 6, the diameter "D5" of the first end portion 724 of the drive shaft 500 is substantially equal to the diameter "D7" and the spindle 780, embodiments in which one of the diameter "D5" and the diameter "D7" is larger than the other are within the scope of the present invention.

Referring also to Figure 4, it may be desirable to cover or shield the coupler 900. In the embodiment illustrated in Figures 4 and 6, a drive guard 910 covers the coupler 900. The drive guard 910 may be generally U-shaped having a curved portion 914 flanked by a pair of substantially linear portions 915 and 916. The distal end of each of the substantially linear portions 915 and 916 of the drive guard 910 may have a flange 918 and 919, respectively. The drive guard 910 may be fastened by each of its flanges 918 and 919 to the base 106.

The motor 510 may be supported on the base 106 by a support member 920. The support member 920 may be coupled to the motor 510 near the spindle 780. In the embodiment depicted, the support member 920 includes a through-hole through which the spindle 780 passes. The support member 920 may be coupled to the motor 510 using any method known in the art, including bolting the support member 920 to the motor 510 with one or more bolts 940.

The coupler 900 may include any coupler suitable for transmitting a sufficient amount of torque from the spindle 780 to the drive shaft 500 to rotate the rotor 600 inside to the stator 700. In the embodiment illustrated in Figures 4 and 6, the coupler 900 is a bellows coupler. A bellows coupler may be beneficial if the spindle 780 and the drive shaft 500 are misaligned. Further, the bellows coupler may help absorb axial forces exerted on the drive shaft 500 that would otherwise be translated to the spindle 780. A suitable bellows coupler includes a model "BC32-8-8-A," manufactured by Ruland Manufacturing Company, Inc. of Marlborough, MA, which operates a website at www.ruland.com.

The motor 510 may rotate the rotor 600 at about 0.1 revolutions per minute ("rpm") to about 7200 rpm. The motor 510 may include any motor suitable for rotating the rotor 600 inside to the stator 700 in accordance with the present teachings. By way of non-limiting example, a suitable motor may include a one-half horsepower electric motor, operating at 230/460 volts and 3450 per minute ("rpm"). A suitable motor includes a model "C4T34NC4C" manufactured by LEESON Electric Corporation of Grafton, WI, which operates a website at www.leeson.com.

FIRST CHAMBER 310

Turning to Figures 4 and 7, the first chamber 320 is disposed inside the central section 522 of the housing 520 between the first mechanical seal housing 524 and the first end portions 612 and 712 of the rotor 600 and the stator 700, respectively. The

first chamber 310 may be annular and have a substantially circular cross-sectional shape. The first chamber 310 and the mixing chamber 330 form a continuous volume. A portion 1020 of the drive shaft 500 extends through the first chamber 310.

As may best be viewed in Figure 4, the first chamber 310 has an input port 1010 through which the first material 110 enters the mixing device 100. The first material 110 may be pumped inside the first chamber 310 by the external pump 210 (see Figure 2). The external pump 210 may include any pump known in the art for pumping the first material 110 at a sufficient rate to supply the first chamber 310.

The input port 1010 is oriented substantially orthogonally to the axis of rotation "a." Therefore, the first material 110 enters the first chamber 310 with a velocity tangential to the portion 1020 of the drive shaft 500 extending through the first chamber 310. The tangential direction of the flow of the first material 110 entering the first chamber 310 is identified by arrow "T1." In the embodiment depicted in Figures 4 and 7, the input port 1010 may be offset from the axis of rotation "a." As is apparent to those of ordinary skill in the art, the direction of the rotation of the drive shaft 500 (identified by arrow "C1" in Figure 9), has a tangential component. The input port 1010 is positioned so that the first material 110 enters the first chamber 310 traveling in substantially the same direction as the tangential component of the direction of rotation of the drive shaft 500.

The first material 110 enters the first chamber 310 and is deflected by the inside of the first chamber 310 about the portion 1020 of the drive shaft 500. In embodiments wherein the first chamber 310 has a substantially circular cross-sectional shape, the inside of the first chamber 310 may deflect the first material 110 in a substantially circular path (identified by arrow "C2" in Figure 9) about the portion 1020 of the drive shaft 500. In such an embodiment, the tangential velocity of the first material 110 may cause it to travel about the axis of rotation "a" at a circumferential velocity, determined at least in part by the tangential velocity.

Once inside the first chamber 310, the first material 110 may be pumped from the first chamber 310 into the mixing chamber 330 by the pump 410 residing inside the first chamber 310. In embodiments that include the external pump 210 (see Figure 2), the external pump 210 may be configured to pump the first material 110 into the first chamber 310 at a rate at least as high as a rate at which the pump 410 pumps the first material 110 from the first chamber 310.

The first chamber 310 is in communication with the open first end portion 332 of the mixing chamber 330 and the first material 110 inside the first chamber 310 may flow freely into the open first end portion 332 of the mixing chamber 330. In this manner, the first material 110 does not negotiate any corners or bends between the mixing chamber 330 and the first chamber 310. In the embodiment depicted, the first chamber 310 is in communication with the entire open first end portion 332 of the mixing chamber 330. The first chamber 310 may be filled completely with the first material 110.

The pump 410 is powered by the portion 1020 of the drive shaft 500 extending through the first chamber 310. The pump 410 may include any pump known in the art having a rotating pump member 2022 housed inside a chamber (i.e., the first chamber 310) defined by a stationary housing (i.e., the housing 520). Non-limiting examples of suitable pumps include rotary positive displacement pumps such as progressive cavity pumps, single screw pumps (e.g., Archimedes screw pump), and the like.

The pump 410 depicted in Figures 7 and 9, is generally referred to as a single screw pump. In this embodiment, the pump member 2022 includes a collar portion 2030 disposed around the portion 1020 of the drive shaft 500. The collar portion 2030 rotates with the portion 1020 of the drive shaft 500 as a unit. The collar portion 2030 includes one or more fluid displacement members 2040. In the embodiment depicted in Figures 7 and 9, the collar portion 2030 includes a single fluid displacement member 2040 having a helical shape that circumscribes the collar portion 2030 along a helical path.

Referring to Figure 9, the inside of the first chamber 310 is illustrated. The pump 410 imparts an axial flow (identified by arrow "A1" and arrow "A2") in the first material 110 inside the first chamber 310 toward the open first end portion 332 of the mixing chamber 330. The axial flow of the first material 110 imparted by the pump 410 has a pressure that may exceed the pressure obtainable by the external pump of the prior art device 10 (see Figure 1).

The pump 410 may also be configured to impart a circumferential flow (identified by arrow "C2") in the first material 110 as it travels toward the open first end portion 332 of the mixing chamber 330. The circumferential flow imparted in the first material 110 before it enters the mixing chamber 330 causes the first material 110 to enter the mixing chamber 330 already traveling in the desired direction at an initial circumferential

velocity. In the prior art device 10 depicted in Figure 1, the first material 110 entered the channel 32 of the prior art device 10 without a circumferential velocity. Therefore, the rotor 12 of the prior art device 10 alone had to impart a circumferential flow into the first material 110. Because the first material 110 is moving axially, in the prior art device 10, the first material 110 traversed at least a portion of the channel 32 formed between the rotor 12 and the stator 30 at a slower circumferential velocity than the first material 110 traverses the mixing chamber 330 of the mixing device 100. In other words, if the axial velocity of the first material 110 is the same in both the prior art device 10 and the mixing device 100, the first material 110 may complete more revolutions around the rotational axis "a" before traversing the axial length of the mixing chamber 330, than it would complete before traversing the axial length of the channel 32. The additional revolutions expose the first material 110 (and combined first material 110 and second material 120) to a substantially larger portion of the effective inside surface 706 (see Figure 7) of the stator 700.

In embodiments including the external pump 210 (see Figure 2), the circumferential velocity imparted by the external pump 210 combined with the input port 1010 being oriented according to the present teachings, may alone sufficiently increase the revolutions of the first material 110 (and combined first material 110 and second material 120) about the rotational axis "a." Further, in some embodiments, the circumferential velocity imparted by the pump 210 and the circumferential velocity imparted by the pump 410 combine to achieve a sufficient number of revolutions of the first material 110 (and combined first material 110 and second material 120) about the rotational axis "a." As is appreciated by those of ordinary skill in the art, other structural elements such as the cross-sectional shape of the first chamber 310 may contribute to the circumferential velocity imparted by the pump 210, the pump 410, and a combination thereof.

In an alternate embodiment depicted in Figure 10, the pump 410 may include one or more vanes 2042 configured to impart a circumferential flow in the first material 110 as it travels toward the open first end portion 332 of the mixing chamber 330.

SECOND CHAMBER 320

Turning now to Figures 4 and 7, the second chamber 320 is disposed inside the central section 522 of the housing 520 between the second mechanical seal housing 526 and the second end portions 614 and 714 of the rotor 600 and the stator 700, respectively. The second chamber 320 may be substantially similar to the first chamber 310. However, instead of the input port 1010, the second chamber 320 may include an output port 3010. A portion 3020 of the drive shaft 500 extends through the second chamber 320.

The second chamber 320 and the mixing chamber 330 form a continuous volume. Further, the first chamber 310, the mixing chamber 330, and the second chamber 320 form a continuous volume. The first material 110 flows through the mixing device 100 from the first chamber 310 to the mixing chamber 330 and finally to the second chamber 320. While in the mixing chamber 330, the first material 110 is mixed with the second material 120 to form the output material 102. The output material 102 exits the mixing device 100 through the output port 3010. Optionally, the output material 102 may be returned to the input port 1010 and mixed with an additional quantity of the second material 120, the third material 130, or a combination thereof.

The output port 3010 is oriented substantially orthogonally to the axis of rotation "a" and may be located opposite the input port 1010 formed in the first chamber 310. The output material 102 enters the second chamber 320 from the mixing chamber 330 having a circumferential velocity (in the direction indicated by arrow "C3" in Figure 9) imparted thereto by the rotor 600. The circumferential velocity is tangential to the portion 3020 of the drive shaft 500 extending through the second chamber 320. In the embodiment depicted in Figures 4, 6, and 7, the output port 3010 may be offset from the axis of rotation "a." The output port 3010 is positioned so that the output material 102, which enters the second chamber 320 traveling in substantially the same direction in which the drive shaft 500 is rotating (identified in Figure 9 by arrow "C1"), is traveling toward the output port 3010.

The output material 102 enters the second chamber 320 and is deflected by the inside of the second chamber 320 about the portion 3020 of the drive shaft 500. In embodiments wherein the second chamber 320 has a substantially circular cross-sectional shape, the inside of the second chamber 320 may deflect the output material 102 in a substantially circular path about the portion 3020 of the drive shaft 500.

Referring to Figure 2, optionally, the output material 102 may be pumped from inside the second chamber 320 by the external pump 430. The external pump 430 may include any pump known in the art for pumping the output material 102 at a sufficient rate to avoid limiting throughput of the mixing device 100. In such an embodiment, the external pump 430 may introduce a tangential velocity (in a direction indicated by arrow "T2" in Figures 4 and 11) to at least a portion of the output material 102 as the external pump 430 pumps the output material 102 from the second chamber 320. The tangential velocity of the portion of the output material 102 may cause it to travel about the axis of rotation "a" at a circumferential velocity, determined in part by the tangential velocity.

PUMP 420

Turning to Figures 6 and 7, the pump 420 residing inside the second chamber 320 may pump the output material 102 from the second chamber 320 into the output port 3010 and/or from the mixing chamber 330 into the second chamber 320. In embodiments that include the external pump 430, the external pump 430 may be configured to pump the output material 102 from the second chamber 320 at a rate at least as high as a rate at which the pump 420 pumps the output material 102 into the output port 3010.

The second chamber 320 is in communication with the open second end portion 334 of the mixing chamber 330 and the output material 102 inside the mixing chamber 330 may flow freely from the open second end portion 334 into the second chamber 320. In this manner, the output material 102 does not negotiate any corners or bends between the mixing chamber 330 and the second chamber 320. In the embodiment depicted, the second chamber 320 is in communication with the entire open second end portion 334 of the mixing chamber 330. The second chamber 320 may be filled completely with the output material 102.

The pump 420 is powered by the portion 3020 of the drive shaft 500 extending through the second chamber 320. The pump 420 may be substantially identical to the pump 410. Any pump described above as suitable for use as the pump 410 may be used for the pump 420. While the pump 410 pumps the first material 110 into the mixing chamber 330, the pump 420 pumps the output material 102 from the mixing chamber 330. Therefore, both the pump 410 and the pump 420 may be oriented to pump in the same direction.

As is appreciated by those of ordinary skill in the art, the first material 110 may differ from the output material 102. For example, one of the first material 110 and the output material 102 may be more viscous than the other. Therefore, the pump 410 may differ from the pump 420. The pump 410 may be configured to accommodate the properties of the first material 110 and the pump 420 may be configured to accommodate the properties of the output material 102.

The pump 420 depicted in Figures 6 and 7, is generally referred to as a single screw pump. In this embodiment, the pump member 4022 includes a collar portion 4030 disposed around the portion 3020 of the drive shaft 500. The collar portion 4030 rotates with the portion 3020 of the drive shaft 500 as a unit. The collar portion 4030 includes one or more fluid displacement members 4040. The collar portion 4030 includes a single fluid displacement member 4040 having a helical shape that circumscribes the collar portion 4030 along a helical path.

Referring to Figure 11, the inside of the second chamber 320 is illustrated. The pump 420 imparts an axial flow (identified by arrow "A3" and arrow "A4") in the output material 102 inside the second chamber 320 away from the open second end portion 334 of the mixing chamber 330.

The pump 420 may be configured to impart a circumferential flow (identified by arrow "C4") in the output material 102 as it travels away from the open second end portion 334 of the mixing chamber 330. The circumferential flow imparted in the output material 102 may help reduce an amount of work required by the rotor 600. The circumferential flow also directs the output material 102 toward the output port 3010.

In an alternate embodiment, the pump 420 may have substantially the same configuration of the pump 410 depicted in Figure 10. In such an embodiment, the one or more vanes 2042 are configured to impart a circumferential flow in the output material 102 as it travels away from the open second end portion 334 of the mixing chamber 330.

As is apparent to those of ordinary skill, various parameters of the mixing device 100 may be modified to obtain different mixing characteristics. Exemplary parameters that may be modified include the size of the through-holes 608, the shape of the through-holes 608, the arrangement of the through-holes 608, the number of through-holes 608, the size of the apertures 708, the shape of the apertures 708, the arrangement of the apertures 708, the number of apertures 708, the shape of the rotor 600, the shape of the stator 700, the width of the mixing chamber 330, the length

of the mixing chamber 330, rotational speed of the drive shaft 500, the axial velocity imparted by the internal pump 410, the circumferential velocity imparted by the internal pump 410, the axial velocity imparted by the internal pump 420, the circumferential velocity imparted by the internal pump 420, the configuration of disturbances (e.g., texture, projections, recesses, apertures, and the like) formed on the outside surface 606 of the rotor 600, the configuration of disturbances (e.g., texture, projections, recesses, apertures, and the like) formed on the inside surface 706 of the stator 700, and the like.

ALTERNATE EMBODIMENT

Referring to Figure 12, a mixing device 5000 is depicted. The mixing device 5000 is an alternate embodiment of the mixing device 100. Identical reference numerals have been used herein to identify components of the mixing device 5000 that are substantially similar corresponding components of the mixing device 100. Only components of the mixing device 5000 that differ from the components of the mixing device 100 will be described.

The mixing device 5000 includes a housing 5500 for housing the rotor 600 and the stator 5700. The stator 5700 may be non-rotatably couple by its first end portion 5712 and its second end portion 5714 to the housing 5500. A chamber 5800 is defined between the housing 5500 and a portion 5820 of the stator 5700 flanked by the first end portion 5712 and the second end portion 5714. The housing 5500 includes an input port 5830 which provides access into the chamber 5800. The input port 5830 may be oriented substantially orthogonally to the axis of rotation "a." however, this is not a requirement.

The stator 5700 includes a plurality of through-holes 5708 that connect the chamber 5800 and the mixing chamber 330 (defined between the rotor 600 and the stator 5700). An external pump 230 may be used to pump the third material 130 (which may be identical to the second material 120) into the chamber 5800 via the input port 5830. The third material 130 pumped into the chamber 5800 may enter the mixing chamber 330 via the through-holes 5708 formed in the stator 5700. The third material 130 may be forced from the chamber 5800 by the pump 230, buoyancy of the third material 130 relative to the first material 110, and a combination thereof. As the rotor 600 rotates, it may also draw the third material 130 from the chamber 5800 into the mixing chamber 330. The third material 130 may enter the mixing chamber 330 as

bubbles, droplets, particles, and the like, which are imparted with a circumferential velocity by the rotor 600.

ALTERNATE EMBODIMENT

An alternate embodiment of the mixing device 100 may be constructed using a central section 5900 depicted in Figure 13 and a bearing housing 5920 depicted in Figure 14. Figure 13 depicts the central section 5900 having in its interior the stator 700 (see Figure 7). Identical reference numerals have been used herein to identify components associated with the central section 5900 that are substantially similar corresponding components of the mixing device 100. Only components of the central section 5900 that differ from the components of the central section 522 will be described. The central section 5900 and the stator 700 are both constructed from a conductive material such as a metal (e.g., stainless steel). The input port 1010 and the output port 3010 are both constructed from a nonconductive material such as plastic (e.g., PET, Teflon, nylon, PVC, polycarbonate, ABS, Delrin, polysulfone, etc.).

An electrical contact 5910 is coupled to the central section 5900 and configured to deliver a charge to thereto. The central section 5900 conducts an electrical charge applied to the electrical contact 5910 to the stator 700. In further embodiments, the central section 5900 may be constructed from a nonconductive material. In such embodiments, the electrical contact 5910 may pass through the central section 5900 and coupled to the stator 700. The electric charge applied by the electrical contact 5910 to the stator 700 may help facilitate redox or other chemical reactions inside the mixing chamber 330.

Optionally, insulation (not shown) may be disposed around the central section 5900 to electrically isolate it from the environment. Further, insulation may be used between the central section 5900 and the first and second mechanical seals 524 and 526 that flank it to isolate it electrically from the other components of the mixing device.

Turning now to Figure 14, the bearing housing 5920 will be described. The bearing housing 5920 is disposed circumferentially around the portion 726 of the drive shaft 500. An electrical contact 5922 is coupled to the bearing housing 5920. A rotating brush contact 5924 provides an electrical connection between the drive shaft 500 and the electrical contact 5922.

In this embodiment, the drive shaft 500 and the rotor 600 are both constructed from a conductive material such as a metal (e.g., stainless steel). The bearing housing 5920 may be constructed from either a conductive or a nonconductive material. An electrical charge is applied to the drive shaft 500 by the electrical contact 5922 and the rotating brush contact 5924. The electrical charge is conducted by the drive shaft 500 to the rotor 600.

The alternate embodiment of the mixing device 100 constructed using the central section 5900 depicted in Figure 13 and the bearing housing 5920 depicted in Figure 14 may be operated in at least two ways. First, the electrical contacts 5910 and 5922 may be configured not to provide an electrical charge to the stator 700 and the rotor 600, respectively. In other words, neither of the electrical contacts 5910 and 5922 are connected to a current source, a voltage source, and the like.

Alternatively, the electrical contacts 5910 and 5922 may be configured to provide an electrical charge to the stator 700 and the rotor 600, respectively. For example, the electrical contacts 5910 and 5922 may be coupled to a DC voltage source (not shown) supplying a steady or constant voltage across the electrical contacts 5910 and 5922. The negative terminal of the DC voltage source may be coupled to either of the electrical contacts 5910 and 5922 and the positive terminal of the DC voltage source may be coupled to the other of the electrical contacts 5910 and 5922. The voltage supplied across the electrical contacts 5910 and 5922 may range from about 0.0001 volts to about 1000 volts. In particular embodiments, the voltage may range from about 1.8 volts to about 2.7 volts. By way of another example, a pulsed DC voltage having a duty cycle of between about 1% to about 99% may be used.

While the above examples of methods of operating the mixing device apply a DC voltage across the electrical contacts 5910 and 5922, as is apparent to those of ordinary skill in the art, a symmetrical AC voltage or non symmetrical AC voltage having various shapes and magnitudes may be applied across the electrical contacts 5910 and 5922 and such embodiments are within the scope of the present invention.

MIXING INSIDE THE MIXING CHAMBER 330

As mentioned above, in the prior art device 10 (shown in Figure 1), the first material 110 entered the channel 32 between the rotor 12 and the stator 30 via a single limited input port 37 located along only a portion of the open second end of the channel 32. Likewise, the output material 102 exited the channel 32 via a single limited

output port 40 located along only a portion of the open first end of the channel 32. This arrangement caused undesirable and unnecessary friction. By replacing the single limited inlet port 37 and the single limited outlet port 40 with the chambers 310 and 320, respectively, friction has been reduced. Moreover, the first material 110 does not negotiate a corner before entering the mixing chamber 330 and the output material 102 does not negotiate a corner before exiting the mixing chamber 330. Further, the chambers 310 and 320 provide for circumferential velocity of the material prior to entering, and after exiting the channel 32.

Accordingly, pressure drop across the mixing device 100 has been substantially reduced. In the embodiments depicted in Figures 2, 4-9, and 11, the pressure drop between the input port 1010 and the output port 3010 is only approximately 12 psi when the mixing device 100 is configured to produce about 60 gallons of the output material 102 per minute. This is an improvement over the prior art device 10 depicted in Figure 1, which when producing about 60 gallons of output material per minute was at least 26 psi. In other words, the pressure drop across the mixing device 100 is less than half that experienced by the prior art device 10.

According to additional aspects, the inclusion of pumps 410 and 420, which are powered by the drive shaft 500, provides a configuration that is substantially more efficient in mixing materials and that requires less energy than the external pumps used in the prior art.

MICRO-CAVITATION

During operation of the mixing device 100, the input materials may include the first material 110 (e.g., a fluid) and the second material 120 (e.g., a gas). The first material 110 and the second material 120 are mixed inside the mixing chamber 330 formed between the rotor 600 and the stator 700. Rotation of the rotor 600 inside the stator 700 agitates the first material 110 and the second material 120 inside the mixing chamber 330. The through-holes 608 formed in the rotor 600 and/or the apertures 708 formed in the stator 700 impart turbulence in the flow of the first material 110 and the second material 120 inside the mixing chamber 330.

Without being limited by theory, the efficiency and persistence of the diffusion of the second material 120 into the first material 110 is believed to be caused in part by micro-cavitation, which is described in connection with Figures 15-17. Whenever a material flows over a smooth surface, a rather laminar flow is established with a thin

boundary layer that is stationary or moving very slowly because of the surface tension between the moving fluid and the stationary surface. The through-holes 608 and optionally, the apertures 708, disrupt the laminar flow and can cause localized compression and decompression of the first material 110. If the pressure during the decompression cycle is low enough, voids (cavitation bubbles) will form in the material. The cavitation bubbles generate a rotary flow pattern 5990, like a tornado, because the localized area of low pressure draws the host material and the infusion material, as shown in Figure 15. When the cavitation bubbles implode, extremely high pressures result. As two aligned openings (e.g., one of the apertures 708 and one of the through-holes 608) pass one another, a succussion (shock wave) occurs, generating significant energy. The energy associated with cavitation and succussion mixes the first material 110 and the second material 120 together to an extremely high degree, perhaps at the molecular level.

The tangential velocity of the rotor 600 and the number of openings that pass each other per rotation may dictate the frequency at which the mixing device 100. It has been determined that operating the mixing device 100 within in the ultrasonic frequency range can be beneficial in many applications. It is believed that operating the mixing device 100 in the ultrasonic region of frequencies provides the maximum succession shock energy to shift the bonding angle of the fluid molecule, which enables it to transport an additional quantity of the second material 120 which it would not normally be able to retain. When the mixing device 100 is used as a diffuser, the frequency at which the mixing device 100 operates appears to affect the degree of diffusion, leading to much longer persistence of the second material 120 (infusion material) in the first material 110 (host material).

Referring now to Figure 15, an alternate embodiment of the rotor 600, rotor 6000 is provided. The cavitations created within the first material 110 in the mixing chamber 330 may be configured to occur at different frequencies along the length of the mixing chamber 330. The frequencies of the cavitations may be altered by altering the number and/or the placement of the through-holes 6608 along the length of the rotor 600. Each of the through-holes 6608 may be substantially similar to the through-holes 608 (discussed above).

By way of non-limiting example, the rotor 6000 may be subdivided into three separate exemplary sections 6100, 6200, and 6300. The through-holes 6608 increase in density from the section 6100 to the section 6200, the number of holes in the

section 6100 being greater than the number of holes in the section 6200. The through-holes 6608 also increase in density from the section 6200 to the section 6300, the number of holes in the section 6200 being greater than the number of holes in the section 6300. Each of the sections 6100, 6200, and 6300 create succussions within their particular area at a different frequency due to the differing numbers of through-holes 6608 formed therein.

By manufacturing the rotor 6000 with a desired number of through-holes 6608 appropriately arranged in a particular area, the desired frequency of the succussions within the mixing chamber 330 may be determined. Similarly, the desired frequency of the cavitations may be determined by a desired number of apertures 708 appropriately arranged in a particular area upon the stator 700 within which the rotor 600 rotates. Further, the desired frequency (or frequencies) of the succussions within the mixing chamber 330 may be achieved by selecting both a particular number and arrangement of the apertures 708 formed in the stator 700 and a particular number and arrangement of the through-holes 608 formed in the rotor 600.

Figures 19-21, depict various alternative arrangements of the apertures 708 formed in the stator 700 and the through-holes 608 formed in the rotor 600 configured to achieve different results with respect to the cavitations created. Figure 16 illustrates a configuration in which the apertures 708 and the through-holes 608 are aligned along an axis 7000 that is not parallel with any line (e.g., line 7010) drawn through the axis of rotation "a" of the rotor 600. In other words, if the rotor 600 has a cylindrical shape, the axis 7000 does not pass through the center of the rotor 600. Thus, the first material 110 within the mixing chamber 330 will not be oriented perpendicularly to the compressions and decompressions created by the apertures 708 and the through-holes 608. The compressions and decompressions will instead have a force vector that has at least a component parallel to the circumferential flow (in the direction of arrow "C3" of Figure 9) of first material 110 within the mixing chamber 330.

Relative alignment of the apertures 708 and the through-holes 608 may also affect the creation of cavitations in the mixing chamber 330. Figure 17 illustrates an embodiment in which the apertures 708 are in registration across the mixing chamber 330 with the through-holes 608. In this embodiment, rotation of the rotor 600 brings the through-holes 608 of the rotor into direct alignment with the apertures 708 of the stator 700. When in direct alignment with each other, the compressive and

decompressive forces created by the apertures 708 and the through-holes 608 are directly aligned with one another.

In the embodiment depicted in Figure 18, the apertures 708 and the through-holes 608 are offset by an offset amount "X" along the axis of rotation "a.". By way of non-limiting example, the offset amount "X" may be determined as a function of the size of the apertures 708. For example, the offset amount "X" may be approximately equal to one half of the diameter of the apertures 708. Alternatively, the offset amount "X" may be determined as a function of the size of the through-holes 608. For example, the offset amount "X" may be approximately equal to one half of the diameter of the through-holes 608. If features (e.g., recesses, projections, etc.) other than or in addition to the through-holes 608 and the apertures 708 are included in either the rotor 600 or the stator 700, the offset amount "X" may be determined as a function of the size of such features. In this manner, the compressive and decompressive forces caused by the apertures 708 of the stator 700 and the through-holes 608 of the rotor 600 collide at a slight offset causing additional rotational and torsional forces within the mixing chamber 330. These additional forces increase the mixing (e.g., diffusive action) of the second material 120 into the first material 110 within the mixing chamber 330.

Referring now to Figures 22-25, non-limiting examples of suitable cross-sectional shapes for the apertures 708 and the through-holes 608 are provided. The cross-sectional shape of the apertures 708 and/or the through-holes 608 may be square as illustrated in Figure 22, circular as illustrated in Figure 23, and the like.

Various cross-sectional shapes of apertures 708 and/or the through-holes 608 may be used to alter flow of the first material 110 as the rotor 600 rotates within the stator 700. For example, Figure 24 depicts a teardrop cross-sectional shape having a narrow portion 7020 opposite a wide portion 7022. If the through-holes 608 have this teardrop shape, when the rotor 600 is rotated (in the direction generally indicated by the arrow "F"), the forces exerted on the first material 110, the second material 120, and optionally the third material 130 within the mixing chamber 330 increase as the materials pass from the wide portion 7022 of the teardrop to the narrow portion 7020.

Additional rotational forces can be introduced into the mixing chamber 330 by forming the apertures 708 and/or the through-holes 608 with a spiral configuration as illustrated in Figure 25. Material that flows into and out of the apertures 708 and/or the through-holes 608 having the spiral configuration experience a rotational force induced

by the spiral configuration. The examples illustrated in Figures 22-25 are provided as non-limiting illustrations of alternate embodiments that may be employed within the mixing device 100. By application of ordinary skill in the art, the apertures 708 and/or the through-holes 608 may be configured in numerous ways to achieve various succussive and agitative forces appropriate for mixing materials within the mixing chamber 330.

DOUBLE LAYER EFFECT

The mixing device 100 may be configured to create the output material 102 by complex and non-linear fluid dynamic interaction of the first material 110 and the second material 120 with complex, dynamic turbulence providing complex mixing that further favors electrokinetic effects (described below). The result of these electrokinetic effects may be observed within the output material 102 as charge redistributions and redox reactions, including in the form of solublized electrons that are stabilized within the output material.

Ionization or dissociation of surface groups and/or adsorption of ions from a liquid cause most solid surfaces in contact with the liquid to become charged. Referring to Figure 26, an electrical double layer ("EDL") 7100 forms around exemplary surface 7110 in contact with a liquid 7120. In the EDL 7100, ions 7122 of one charge (in this case, negatively charged ions) adsorb to the surface 7120 and form a surface layer 7124 typically referred to as a Stern layer. The surface layer 7124 attracts counterions 7126 (in this case, positively charged ions) of the opposite charge and equal magnitude, which form a counterion layer 7128 below the surface layer 7124 typically referred to as a diffuse layer. The counterion layer 7128 is more diffusely distributed than the surface layer 7124 and sits upon a uniform and equal distribution of both ions in the bulk material 7130 below. For OH⁻ and H⁺ ions in neutral water, the Gouy-Chapman model would suggest that the diffuse counterion layer extends about one micron into the water.

According to particular aspects, the electrokinetic effects mentioned above are caused by the movement of the liquid 7120 next to the charged surface 7110. Within the liquid 7120 (e.g., water, saline solution, and the like), the adsorbed ions 7122 forming the surface layer 7124 are fixed to the surface 7120 even when the liquid 7120 is in motion (for example, flowing in the direction indicated by arrow "G"); however, a shearing plane 7132 exists within the diffuse counterion layer 7128 spaced from the

surface 7120. Thus, as the liquid 7120 moves, some of the diffuse counterions 7126 are transported away from the surface 7120, while the absorbed ions 7122 remain at the surface 7120. This produces a so-called 'streaming current.'

Within the mixing chamber 330, the first material 110, the second material 120, and optionally, the third material 130 are subject to an electromagnetic field created by the inside surface 705 of the stator 700 and/or the outside surface 606 of the rotor 600, a voltage between the inside surface 705 and the outside surface 606, and/or an electrokinetic effect (e.g., streaming current) caused by at least one EDL formed in the first material 110. The at least one EDL may be introduced into the first material 110 by at least one of the inside surface 705 of the stator 700 and the outside surface 606 of the rotor 600.

Movement of the first material 110 through the mixing chamber 330 relative to surface disturbances (e.g., the through-holes 608 and apertures 708) creates cavitations in the first material 110 within the mixing chamber 330, which may diffuse the second material 120 into the first material 110. These cavitations may enhance contact between of the first material 110 and/or the second material 120 with the electric double layer formed on the inside surface 705 of the stator 700 and/or the electric double layer formed on the outside surface 606 of the rotor 600. Larger surface to volume ratios of the mixing chamber, an increased dwell time of the combined materials within the mixing chamber, and further in combination with a small average bubble size (and hence substantially greater bubble surface area) provide for effectively imparting EDL-mediated effects to the inventive output materials.

In embodiments in which the inside surface 705 and the outside surface 606 are constructed from a metallic material, such as stainless steel, the motion of the liquid 7120 and/or the streaming current(s) facilitate redox reactions involving H_2O , OH^- , H^+ , and O_2 at the inside surface 705 and the outside surface 606.

Referring to Figure 27, without being limited by theory, it is believed a section 7140 of the mixing chamber 330 between the inside surface 705 and the outside surface 606 may be modeled as a pair of parallel plates 7142 and 7144. If the first material 110 is a liquid, the first material 110 enters the section 7140 through an inlet "IN" and exits the section 7140 through an outlet "OUT." The inlet "IN" and the outlet "OUT" restrict the flow into and out of the section 7140.

Referring to Figure 28, the area between the parallel plates 7142 and 7144 has a high surface area to volume ratio. Hence, a substantial portion of the counterion

layer 7128 (and counterions 7126) may be in motion as the first material 110 moves between the plates 7142 and 7144. The number of counterions 7126 in motion may exceed the number allowed to enter the section 7140 by the inlet "IN" and the number allowed to exit the section 7140 by the outlet "OUT." The inlet "IN" and the outlet "OUT" feeding and removing the first material 110 from the section 7140, respectively, have far less surface area (and a lower surface area to volume ratio) than the parallel plates 7142 and 7144 and thereby reduce the portion of the counterions 7126 in motion in the first material 110 entering and leaving the section 7140. Therefore, entry and exit from the section 7140 increases the streaming current locally. While a background streaming current (identified by arrow "BSC") caused by the flowing first material 110 over any surface is always present inside the mixing device 100, the plates 7142 and 7144 introduce an increased "excess" streaming current (identified by arrow "ESC") within the section 7140.

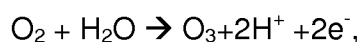
Without a conductive return current (identified by arrow "RC") in the plates 7142 and 7144 in the opposite direction of the flow of the first material 110, an excess charge 7146 having the same sign as the adsorbing ions 7122 would accumulate near the inlet "IN," and an excess charge 7148 having the same sign as the counterion 7126 would accumulate near the at outlet "OUT." Because such accumulated charges 7146 and 7148, being opposite and therefore attracted to one another, cannot build up indefinitely the accumulated charges seek to join together by conductive means. If the plates 7142 and 7144 are perfectly electrically insulating, the accumulated charges 7146 and 7148 can relocate only through the first material 110 itself. When the conductive return current (identified by arrow "RC") is substantially equivalent to the excess streaming current (identified by arrow "ESC") in the section 7140, a steady-state is achieved having zero net excess streaming current, and an electrostatic potential difference between the excess charge 7146 near the inlet "IN," and the excess charge 7148 near the outlet "OUT" creating a steady-state charge separation therebetween.

The amount of charge separation, and hence the electrostatic potential difference between the excess charge 7146 near the inlet "IN," and the excess charge 7148 near the outlet "OUT," depends on additional energy per unit charge supplied by a pump (e.g., the rotor 600, the internal pump 410, and/or the external pump 210) to "push" charge against the opposing electric field (created by the charge separation) to produce the a liquid flow rate approximating a flow rate obtainable by a

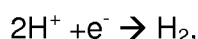
liquid without ions (i.e., ions 7122 and 7126). If the plates 7142 and 7144 are insulators, the electrostatic potential difference is a direct measure of the EMF the pump (e.g., the rotor 600, the internal pump 410 and/or the external pump 210) can generate. In this case, one could measure the electrostatic potential difference using a voltmeter having a pair of leads by placing one of the leads in the first material 110 near the inlet "IN," and the other lead in the first material 110 near the outlet "OUT."

With insulating plates 7142 and 7144, any return current is purely an ion current (or flow of ions), in that the return current involves only the conduction of ions through the first material 110. If other conductive mechanisms through more conductive pathways are present between the excess charge 7146 near the inlet "IN," and the excess charge 7148 near the outlet "OUT," the return current may use those more conductive pathways. For example, conducting metal plates 7142 and 7144 may provide more conductive pathways; however, these more conductive pathways transmit only an electron current and not the ion current.

As is appreciated by those of ordinary skill, to transfer the charge carried by an ion to one or more electrons in the metal, and vice versa, one or more oxidation-reduction reactions must occur at the surface of the metal, producing reaction products. Assuming the first material 110 is water (H₂O) and the second material 120 is oxygen (O₂), a non-limiting example of a redox reaction, which would inject negative charge into the conducting plates 7142 and 7144 includes the following known half-cell reaction:



Again, assuming the first material 110 is water (H₂O) and the second material 120 is oxygen (O₂), a non-limiting example of a redox reaction includes the following known half-cell reaction, which would remove negative charge from the conducting plates 7142 and 7144 includes the following known half-cell reaction:



With conducting metal plates 7142 and 7144, most of the return current is believed to be an electron current, because the conducting plates 7142 and 7144 are more conductive than the first material 110 (provided the redox reactions are fast enough not to be a limiting factor). For the conducting metal plates 7142 and 7144, a smaller charge separation accumulates between the inlet "IN" and the outlet "OUT," and a much smaller electrostatic potential exists therebetween. However, this does not mean that the EMF is smaller.

As described above, the EMF is related to the energy per unit charge the pump provides to facilitate the flow of the first material 110 against the opposing electric field created by the charge separation. Because the electrostatic potential is smaller, the pump may supply less energy per unit charge to cause the first material 110 to flow. However, the above example redox reactions do not necessarily occur spontaneously, and thus may require a work input, which may be provided by the pump. Therefore, a portion of the EMF (that is not reflected in the smaller electrostatic potential difference) may be used to provide the energy necessary to drive the redox reactions.

In other words, the same pressure differentials provided by the pump to push against the opposing electric field created by the charge separation for the insulating plates 7142 and 7144, may be used both to “push” the charge through the conducting plates 7142 and 7144 and drive the redox reactions.

Referring to Figure 29, an experimental setup for an experiment conducted by the inventors is provided. The experiment included a pair of substantially identical spaced apart 500 ml standard Erlenmeyer flasks 7150 and 7152, each containing a volume of deionized water 7153. A rubber stopper 7154 was inserted in the open end of each of the flasks 7150 and 7152. The stopper 7154 included three pathways, one each for a hollow tube 7156, a positive electrode 7158, and a negative electrode 7160. With respect to each of the flasks 7150 and 7152, each of the hollow tube 7156, the positive electrode 7158, and the negative electrode 7160 all extended from outside the flask, through the stopper 7154, and into the deionized water 7153 inside the flask. The positive electrode 7158 and the negative electrode 7160 were constructed from stainless steel. The hollow tubes 7156 in both of the flasks 7150 and 7152 had an open end portion 7162 coupled to a common oxygen supply 7164. The positive electrode 7158 and the negative electrode 7160 inserted into the flask 7152 where coupled to a positive terminal and a negative terminal, respectively, of a DC power supply 7168. Exactly the same sparger was used in each flask.

Oxygen flowed through the hollow tubes 7156 into both of the flasks 7150 and 7152 at a flow rate (Feed) of about 1 SCFH to about 1.3 SCFH (combined flow rate). The voltage applied across the positive electrode 7158 and the negative electrode 7160 inserted into the flask 7152 was about 2.55 volts. This value was chosen because it is believed to be an electrochemical voltage value sufficient to affect all oxygen species. This voltage was applied continuously over three to four hours

during which oxygen from the supply 7164 was bubbled into the deionized water 7153 in each of the flasks 7150 and 7152.

Testing of the deionized water 7153 in the flask 7150 with HRP and pyrogallol gave an HRP-mediated pyrogallol reaction activity, consistent with the properties of fluids produced with the alternate rotor/stator embodiments described herein. The HRP optical density was about 20% higher relative to pressure-pot or fine-bubbled solutions of equivalent oxygen content. The results of this experiment indicate that mixing inside the mixing chamber 330 involves a redox reaction. According to particular aspects, the inventive mixing chambers provide for output materials comprising added electrons that are stabilized by either oxygen-rich water structure within the inventive output solutions, or by some form of oxygen species present due to the electrical effects within the process.

Additionally, the deionized water 7153 in both of the flasks 7150 and 7152 was tested for both ozone and hydrogen peroxide employing industry standard colorimetric test ampoules with a sensitivity of 0.1 ppm for hydrogen peroxide and 0.6 ppm for ozone. There was no positive indication of either species up to the detection limits of those ampoules.

DWELL TIME

Dwell time is an amount of time the first material 110, the second material 120, and optionally the third material 130 spend in the mixing chamber 330. The ratio of the length of the mixing chamber 330 to the diameter of the mixing chamber 330 may significantly affect dwell time. The greater the ratio, the longer the dwell time. As mentioned in the Background Section, the rotor 12 of the prior art device 10 (see Figure 1) had a diameter of about 7.500 inches and a length of about 6.000 inches providing a length to diameter ratio of about 0.8. In contrast, in particular embodiments, the length of the mixing chamber 330 of the mixing device 100 is about 5 inches and the diameter "D1" of the rotor 600 is about 1.69 inches yielding a length to diameter ratio of about 2.95.

Dwell time represents the amount of time that the first material 110, the second material 120, and optionally the third material 130 are able to interact with the electrokinetic phenomena described herein. The prior art device 10 is configured to produce about 60 gallons of the output material 102 per minute and the mixing device 100 is configured to produce about 0.5 gallons of the output material 102 per

minute, the prior art device 10 (see Figure 1) had a fluid dwell time of about 0.05 seconds, whereas embodiments of the mixing device 100 have a substantially greater (about 7-times greater) dwell time of about 0.35 seconds. This longer dwell time allows the first material 110, the second material 120, and optionally the third material 130 to interact with each other and the surfaces 606 and 705 (see Figure 7) inside the mixing chamber 330 for about 7-times longer than was possible in the prior art device 10. In additional embodiments, the dwell time is at least 1.5-times, at least 2-times, at least 3-times, at least 4-times, at least 5-times, at least 6-times, at least 7-times or greater, than was possible in the prior art device 10.

With reference to Table I below, the above dwell times were calculated by first determining the flow rate for each device in gallons per second. In the case of the prior art device 10 was configured to operate at about 60 gallons of output material per minute, while the mixing device 100 is configured to operate over a broader range of flow rate, including at an optimal range of about 0.5 gallons of output material per minute. The flow rate was then converted to cubic inches per second by multiplying the flow rate in gallons per second by the number of cubic inches in a gallon (i.e., 231 cubic inches). Then, the volume (12.876 cubic inches) of the channel 32 of the prior art device 10 was divided by the flow rate of the device (231 cubic inches/second) to obtain the dwell time (in seconds) and the volume (0.673 cubic inches) of the mixing chamber 330 of the mixing device 100 was divided by the flow rate (1.925 cubic inches/second) of the device (in cubic inches per second) to obtain the dwell time (in seconds).

TABLE 1. Inventive device can accommodate a range of dwell times, including a substantially increased (e.g., 7-times) dwell time relative to prior art devices.

Device	Flow Rate Gallons/ Minute	Flow Rate Gallons/ Second	Flow Rate Cubic Inches/ Second	Volume Mixing Chamber (Cubic Inches)	Dwell Time (Seconds)
Prior art device 10	60	1.000	231.000	12.876	0.056
Mixing device 100	2	0.033	7.700	0.673	0.087
Mixing device 100	0.5	0.008	1.925	0.673	0.350

Table I

RATE OF INFUSION

Particular aspects of the mixing device 100 provide an improved oxygen infusion rate over the prior art, including over prior art device 10 (see Figure 1). When the first material 110 is water and the second material 120 is oxygen, both of which are processed by the mixing device 100 in a single pass (i.e., the return block of Figure 2 is set to "NO") at or near 20° Celsius, the output material 102 has a dissolved oxygen level of about 43.8 parts per million. In certain aspects, an output material having about 43.8 ppm dissolved oxygen is created in about 350 milliseconds via the inventive flow through the inventive non pressurized (non-pressure pot) methods. In contrast, when the first material 110 (water) and the second material 120 (oxygen) are both processed in a single pass at or near 20° Celsius by the prior art device 10, the output material had dissolved oxygen level of only 35 parts per million in a single pass of 56 milliseconds.

OUTPUT MATERIAL 102

When the first material 110 is a liquid (e.g., freshwater, saline, GATORADE®, and the like) and the second material 120 is a gas (e.g., oxygen, nitrogen, and the like), the mixing device 100 may diffuse the second material 120 into the first material 110. The following discusses results of analyses performed on the output material 102 to characterize one or more properties of the output material 102 derived from having been processed by the mixing device 100.

When the first material 110 is saline solution and the second material 120 is oxygen gas, experiments have indicated that a vast majority of oxygen bubbles produced within the saline solution are no greater than 0.1 micron in size.

DECAY OF DISSOLVED OXYGEN LEVELS

Referring now to Figure 30, there is illustrated the DO levels in water processed with oxygen in the mixing device 100 and stored in a 500 ml thin-walled plastic bottle and a 1000 ml glass bottle. Each of the bottles was capped and stored at 65 degrees Fahrenheit. Point 7900 is the DO level at bottling. Line 7902 illustrates the Henry's Law equilibrium state (i.e., the amount of dissolved oxygen that should be within the water at 65 degrees Fahrenheit), which is a DO level of slightly less than 10 ppm. Points 7904 and 7906 represent the DO levels within the water in the plastic bottle at 65

days and 95 days respectively. As can be seen at point 7904, when the plastic bottle is opened approximately 65 days after bottling, the DO level within the water is approximately 27.5 ppm. When the bottle is opened approximately 95 days after bottling, as indicated at point 7906, the DO level is approximately 25 ppm. Likewise, for the glass bottle, the DO level is approximately 40 ppm at 65 days as indicated at point 7908 and is approximately 41 ppm at 95 days as illustrated at point 7910. Thus, Figure 30 indicates the DO levels within both the plastic bottle and the glass bottle remain relatively high at 65 degrees Fahrenheit.

Referring now to Figure 31, there is illustrated the DO levels in water enriched with oxygen in the mixing device 100 and stored in a 500 ml thin-walled plastic bottle and a 1000 ml glass bottle out to at least 365 days. Each of the bottles was capped and stored at 65 degrees Fahrenheit. As can be seen in the Figure, the DO levels of the oxygen-enriched fluid remained fairly constant out to at least 365 days.

Referring to Figure 33, there is illustrated the DO levels in water enriched with oxygen in the mixing device 100 and stored in a 500 ml plastic thin-walled bottle and a 1000 ml glass bottle. Both bottles were refrigerated at 39 degrees Fahrenheit. Again, DO levels of the oxygen-enriched fluid remained steady and decreased only slightly out to at least 365 days.

Routes and Forms of Administration

As used herein, "subject," may refer to any living creature, preferably an animal, more preferably a mammal, and even more preferably a human.

In particular exemplary embodiments, the gas-enriched fluid of the present invention may function as a therapeutic composition alone or in combination with another therapeutic agent such that the therapeutic composition prevents or alleviates at least one symptom of a G-Protein Receptor associated disorder. The therapeutic compositions of the present invention include compositions that are able to be administered to a subject in need thereof. In certain embodiments, the therapeutic composition formulation may also comprise at least one additional agent selected from the group consisting of: carriers, adjuvants, emulsifying agents, suspending agents, sweeteners, flavorings, perfumes, and binding agents.

As used herein, "pharmaceutically acceptable carrier" and "carrier" generally refer to a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Some non-limiting examples of materials which can

serve as pharmaceutically acceptable carriers are sugars such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols; such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator. In particular aspects, such carriers and excipients may be gas-enriched fluids or solutions of the present invention.

The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, or diluents, are well known to those who are skilled in the art. Typically, the pharmaceutically acceptable carrier is chemically inert to the therapeutic agents and has no detrimental side effects or toxicity under the conditions of use. The pharmaceutically acceptable carriers can include polymers and polymer matrices, nanoparticles, microbubbles, and the like.

In addition to the therapeutic gas-enriched fluid of the present invention, the therapeutic composition may further comprise inert diluents such as additional non-gas-enriched water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. As is appreciated by those of ordinary skill, a novel and improved formulation of a particular therapeutic composition, a novel gas-enriched therapeutic fluid, and a novel method of delivering the novel gas-enriched therapeutic fluid may be obtained by replacing one or more inert diluents with a gas-enriched fluid of identical, similar, or different composition. For example, conventional water may be replaced or

supplemented by a gas-enriched fluid produced by mixing oxygen into water or deionized water to provide gas-enriched fluid.

In certain embodiments, the inventive gas-enriched fluid may be combined with one or more therapeutic agents and/or used alone. In particular embodiments, incorporating the gas-enriched fluid may include replacing one or more solutions known in the art, such as deionized water, saline solution, and the like with one or more gas-enriched fluid, thereby providing an improved therapeutic composition for delivery to the subject.

Certain embodiments provide for therapeutic compositions comprising a gas-enriched fluid of the present invention, a pharmaceutical composition or other therapeutic agent or a pharmaceutically acceptable salt or solvate thereof, and at least one pharmaceutical carrier or diluent. These pharmaceutical compositions may be used in the prophylaxis and treatment of the foregoing diseases or conditions and in therapies as mentioned above. Preferably, the carrier must be pharmaceutically acceptable and must be compatible with, i.e. not have a deleterious effect upon, the other ingredients in the composition. The carrier may be a solid or liquid and is preferably formulated as a unit dose formulation, for example, a tablet that may contain from 0.05 to 95% by weight of the active ingredient.

Possible administration routes include oral, sublingual, buccal, parenteral (for example subcutaneous, intramuscular, intra-arterial, intraperitoneally, intracisternally, intravesically, intrathecally, or intravenous), rectal, topical including transdermal, intravaginal, intraocular, intraotical, intranasal, inhalation, and injection or insertion of implantable devices or materials.

Administration Routes

Most suitable means of administration for a particular subject will depend on the nature and severity of the disease or condition being treated or the nature of the therapy being used, as well as the nature of the therapeutic composition or additional therapeutic agent. In certain embodiments, oral or topical administration is preferred.

Formulations suitable for oral administration may be provided as discrete units, such as tablets, capsules, cachets, syrups, elixirs, chewing gum, "lollipop" formulations, microemulsions, solutions, suspensions, lozenges, or gel-coated ampules, each containing a predetermined amount of the active compound; as powders or granules;

as solutions or suspensions in aqueous or non-aqueous liquids; or as oil-in-water or water-in-oil emulsions.

Formulations suitable for transmucosal methods, such as by sublingual or buccal administration include lozenges patches, tablets, and the like comprising the active compound and, typically a flavored base, such as sugar and acacia or tragacanth and pastilles comprising the active compound in an inert base, such as gelatin and glycerine or sucrose acacia.

Formulations suitable for parenteral administration typically comprise sterile aqueous solutions containing a predetermined concentration of the active gas-enriched fluid and possibly another therapeutic agent; the solution is preferably isotonic with the blood of the intended recipient. Additional formulations suitable for parenteral administration include formulations containing physiologically suitable co-solvents and/or complexing agents such as surfactants and cyclodextrins. Oil-in-water emulsions may also be suitable for formulations for parenteral administration of the gas-enriched fluid. Although such solutions are preferably administered intravenously, they may also be administered by subcutaneous or intramuscular injection.

Formulations suitable for urethral, rectal or vaginal administration include gels, creams, lotions, aqueous or oily suspensions, dispersible powders or granules, emulsions, dissolvable solid materials, douches, and the like. The formulations are preferably provided as unit-dose suppositories comprising the active ingredient in one or more solid carriers forming the suppository base, for example, cocoa butter. Alternatively, colonic washes with the gas-enriched fluids of the present invention may be formulated for colonic or rectal administration.

Formulations suitable for topical, intraocular, intraotic, or intranasal application include ointments, creams, pastes, lotions, pastes, gels (such as hydrogels), sprays, dispersible powders and granules, emulsions, sprays or aerosols using flowing propellants (such as liposomal sprays, nasal drops, nasal sprays, and the like) and oils. Suitable carriers for such formulations include petroleum jelly, lanolin, polyethyleneglycols, alcohols, and combinations thereof. Nasal or intranasal delivery may include metered doses of any of these formulations or others. Likewise, intraotic or intraocular may include drops, ointments, irrigation fluids and the like.

Formulations of the invention may be prepared by any suitable method, typically by uniformly and intimately admixing the gas-enriched fluid optionally with an active

compound with liquids or finely divided solid carriers or both, in the required proportions and then, if necessary, shaping the resulting mixture into the desired shape.

For example a tablet may be prepared by compressing an intimate mixture comprising a powder or granules of the active ingredient and one or more optional ingredients, such as a binder, lubricant, inert diluent, or surface active dispersing agent, or by molding an intimate mixture of powdered active ingredient and a gas-enriched fluid of the present invention.

Suitable formulations for administration by inhalation include fine particle dusts or mists which may be generated by means of various types of metered dose pressurized aerosols, nebulisers, or insufflators. In particular, powders or other compounds of therapeutic agents may be dissolved or suspended in a gas-enriched fluid of the present invention.

For pulmonary administration via the mouth, the particle size of the powder or droplets is typically in the range 0.5-10 μM , preferably 1-5 μM , to ensure delivery into the bronchial tree. For nasal administration, a particle size in the range 10-500 μM is preferred to ensure retention in the nasal cavity.

Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of a therapeutic agent in a liquefied propellant. In certain embodiments, as disclosed herein, the gas-enriched fluids of the present invention may be used in addition to or instead of the standard liquefied propellant. During use, these devices discharge the formulation through a valve adapted to deliver a metered volume, typically from 10 to 150 μL , to produce a fine particle spray containing the therapeutic agent and the gas-enriched fluid. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures thereof.

The formulation may additionally contain one or more co-solvents, for example, ethanol surfactants, such as oleic acid or sorbitan trioleate, anti-oxidants and suitable flavoring agents. Nebulisers are commercially available devices that transform solutions or suspensions of the active ingredient into a therapeutic aerosol mist either by means of acceleration of a compressed gas (typically air or oxygen) through a narrow venturi orifice, or by means of ultrasonic agitation. Suitable formulations for use in nebulisers consist of another therapeutic agent in a gas-enriched fluid and comprising up to 40% w/w of the formulation, preferably less than 20% w/w. In

addition, other carriers may be utilized, such as distilled water, sterile water, or a dilute aqueous alcohol solution, preferably made isotonic with body fluids by the addition of salts, such as sodium chloride. Optional additives include preservatives, especially if the formulation is not prepared sterile, and may include methyl hydroxy-benzoate, anti-oxidants, flavoring agents, volatile oils, buffering agents and surfactants.

Suitable formulations for administration by insufflation include finely comminuted powders that may be delivered by means of an insufflator or taken into the nasal cavity in the manner of a snuff. In the insufflator, the powder is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened in situ and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the active ingredient or of a powder blend comprising the active ingredient, a suitable powder diluent, such as lactose, and an optional surfactant. The active ingredient typically comprises from 0.1 to 100 w/w of the formulation.

In addition to the ingredients specifically mentioned above, the formulations of the present invention may include other agents known to those skilled in the art, having regard for the type of formulation in issue. For example, formulations suitable for oral administration may include flavoring agents and formulations suitable for intranasal administration may include perfumes.

The therapeutic compositions of the invention can be administered by any conventional method available for use in conjunction with pharmaceutical drugs, either as individual therapeutic agents or in a combination of therapeutic agents.

The dosage administered will, of course, vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent and its mode and route of administration; the age, health and weight of the recipient; the nature and extent of the symptoms; the kind of concurrent treatment; the frequency of treatment; and the effect desired. A daily dosage of active ingredient can be expected to be about 0.001 to 1000 milligrams (mg) per kilogram (kg) of body weight, with the preferred dose being 0.1 to about 30 mg/kg.

Dosage forms (compositions suitable for administration) contain from about 1 mg to about 500 mg of active ingredient per unit. In these pharmaceutical compositions, the active ingredient will ordinarily be present in an amount of about 0.5-95% weight based on the total weight of the composition.

Ointments, pastes, foams, occlusions, creams and gels also can contain excipients, such as starch, tragacanth, cellulose derivatives, silicones, bentonites, silica acid, and talc, or mixtures thereof. Powders and sprays also can contain excipients such as lactose, talc, silica acid, aluminum hydroxide, and calcium silicates, or mixtures of these substances. Solutions of nanocrystalline antimicrobial metals can be converted into aerosols or sprays by any of the known means routinely used for making aerosol pharmaceuticals. In general, such methods comprise pressurizing or providing a means for pressurizing a container of the solution, usually with an inert carrier gas, and passing the pressurized gas through a small orifice. Sprays can additionally contain customary propellants, such as nitrogen, carbon dioxide, and other inert gases. In addition, microspheres or nanoparticles may be employed with the gas-enriched therapeutic compositions or fluids of the present invention in any of the routes required to administer the therapeutic compounds to a subject.

The injection-use formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, or gas-enriched fluid, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets. The requirements for effective pharmaceutical carriers for injectable compositions are well known to those of ordinary skill in the art. See, for example, *Pharmaceutics and Pharmacy Practice*, J. B. Lippincott Co., Philadelphia, Pa., Banker and Chalmers, Eds., 238-250 (1982) and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., 622-630 (1986).

Formulations suitable for topical administration include lozenges comprising a gas-enriched fluid of the invention and optionally, an additional therapeutic and a flavor, usually sucrose and acacia or tragacanth; pastilles comprising a gas-enriched fluid and optional additional therapeutic agent in an inert base, such as gelatin and glycerin, or sucrose and acacia; and mouth washes or oral rinses comprising a gas-enriched fluid and optional additional therapeutic agent in a suitable liquid carrier; as well as creams, emulsions, gels and the like.

Additionally, formulations suitable for rectal administration may be presented as suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. Formulations suitable for vaginal administration may be presented as

pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, Mack Publishing Company, a standard reference text in this field.

The dose administered to a subject, especially an animal, particularly a human, in the context of the present invention should be sufficient to effect a therapeutic response in the animal over a reasonable time frame. One skilled in the art will recognize that dosage will depend upon a variety of factors including the condition of the animal, the body weight of the animal, as well as the condition being treated. A suitable dose is that which will result in a concentration of the therapeutic composition in a subject that is known to affect the desired response.

The size of the dose also will be determined by the route, timing and frequency of administration as well as the existence, nature, and extent of any adverse side effects that might accompany the administration of the therapeutic composition and the desired physiological effect.

The following examples are meant to be illustrative only and not limiting in any way.

EXAMPLES

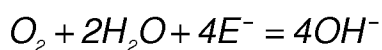
EXAMPLE 1

Generation of Solvated Electrons

Additional evidence has also indicated that the enriching process generated by the diffuser device of the present invention results in solvated electrons within the gas-enriched fluid. Due to the results of the polarographic dissolved oxygen probes, it is believed that the diffused fluid exhibits an electron capture effect and thus the fluid included solvated electrons within the gas-enriched material.

There are two fundamental techniques for measuring dissolved oxygen levels electrically: galvanic measuring techniques and polarographic measurements. Each process uses an electrode system wherein the dissolved oxygen levels within the solution being tested react with a cathode of the probe to produce a current. Dissolved oxygen level sensors consist of two electrodes, an anode and a cathode, which are both immersed in electrolyte within the sensor body. An oxygen permeable membrane

separates the anode and cathode from the solution being tested. Oxygen diffuses across the membrane and interacts with the internal components of the probe to produce an electrical current. The cathode is a hydrogen electrode and carries negative potential with respect to the anode. The electrolyte solution surrounds the electrode pair and is contained by the membrane. When no oxygen is present, the cathode is polarized by hydrogen and resists the flow of current. When oxygen passes through the membrane, the cathode is depolarized and electrons are consumed. The cathode electrochemically reduces the oxygen to hydroxyl ions according to the following equation:



When performing dissolved oxygen level measurements of a gas-enriched solution according to the systems of the present invention, an overflow condition has been repeatedly experienced wherein the dissolved oxygen meter displays a reading that is higher than the meter is capable of reading. However, evaluation of the gas-enriched solution by Winkler Titration indicates lower dissolved oxygen (DO) level for the solution than indicated by the probe. Typically, a DO probe (such as the Orion 862 used in these experiments) has a maximum reading of 60 ppm. However, when the meter is left in gas-enriched water of the present invention, it overflows.

Without wishing to be bound by any particular mechanism of action, the mechanism of the meter responds to electrons where the oxygen reacts. However, according to electron spin resonance, no free ions are present in the fluid. Thus, the fluid presumably contains solvated electrons stabilized by the oxygen species that is also present in the fluid.

EXAMPLE 2

Glutathione Peroxidase Study

The inventive oxygen-enriched fluid was tested for the presence of hydrogen peroxide by testing the reactivity with glutathione peroxidase using a standard assay (Sigma). Water samples were tested by adding the enzyme cocktail and inverting. Continuous spectrophotometric rate determination was made at A_{340} nm, and room temperature (25 degrees Celsius). Samples tested were: 1. deionized water (negative control), 2. inventive oxygen-enriched fluid at low concentration, 3. inventive oxygen-enriched fluid at high concentration, 4. hydrogen peroxide (positive control). The

hydrogen peroxide positive control showed a strong reactivity, while none of the other fluids tested reacted with the glutathione peroxidase.

EXAMPLE 3

(Electrokinetically generated superoxygenated fluids and Solas were shown to provide for synergistic prolongation effects (e.g., suppression of bronchoconstriction) with Albuterol in vivo in an art-recognized animal model of human bronchoconstriction (human asthma model))

Experiment 1:

In an initial experiment, sixteen guinea pigs were evaluated for the effects of bronchodilators on airway function in conjunction with methacholine-induced bronchoconstriction. Following determination of optimal dosing, each animal was dosed with 50 µg/mL to deliver the target dose of 12.5 µg of albuterol sulfate in 250 µL per animal.

The study was a randomized blocked design for weight and baseline PenH values. Two groups (A and B) received an intratracheal instillation of 250 µL of 50 µg/mL albuterol sulfate in one or two diluents: Group A was deionized water that had passed through the inventive device, without the addition of oxygen, while Group B was inventive gas-enriched water. Each group was dosed intratracheally with solutions using a Penn Century Microsprayer. In addition, the animals were stratified across BUXCO plethysmograph units so that each treatment group is represented equally within nebulizers feeding the plethysmographs and the recording units.

Animals that displayed at least 75% of their baseline PenH value at 2 hours following albuterol administration were not included in the data analyses. This exclusion criteria is based on past studies where the failure to observe bronchoprotection with bronchodilators can be associated with dosing errors. As a result, one animal from the control group was dismissed from the data analyses.

Once an animal had greater than 50% bronchoconstriction, the animal was considered to be not protected. As set forth in Table 3 below, 50% of the Group B animals (shaded) were protected from bronchoconstriction out to 10 hours (at which time the test was terminated).

Table 3

Bronchoconstriction Protection as Measured with Methacholine Challenge

Group A

Percent protection from bronchoconstriction by animal number

Time (hours)	1	2	3	4	5	6	7
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	20.81	23.82	32.89	11.56	7.91	24.95	20.15
6	15.67	9.96	8.53	8.40	81.66	75.60	91.97
10	173.92	130.34	95.45	68.14	57.85	103.95	69.03

Group B

Percent protection from bronchoconstriction by animal number

Time (hours)	1	2	3	4	5	6	7	8
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	15.85	18.03	17.88	24.09	18.59	15.18	21.33	13.33
6	211.57	10.96	68.79	23.72	11.09	99.00	118.26	6.95
10	174.54	12.87	88.16	20.40	21.45	31.60	123.47	8.46

Experiment 2: A Bronchoconstriction Evaluation of RDC1676 With Albuterol Sulfate in Male Hartley Guinea Pigs:

An additional set of experiments was conducted using a larger number of animals to evaluate the protective effects of the inventive electrokinetically generated fluids (e.g, RDC1676-00, RDC1676-01, RDC1676-02 and RDC1676-03) against methacholine-induced bronchoconstriction when administered alone or as diluents for albuterol sulfate in male guinea pigs.

Materials:

Guinea Pigs (*Cavia porcellus*) were Hartley albino, CrI:(HA)BR from Charles River Canada Inc. (St. Constant, Quebec, Canada). Weight: Approximately 325 ± 50 g at the onset of treatment. Number of groups was 32, with 7 male animals per group (plus 24 spares from same batch of animals). Diet; All animals had free access to a standard certified pelleted commercial laboratory diet (PMI Certified Guinea Pig 5026; PMI Nutrition International Inc.) except during designated procedures.

Methods:

Route of administration was intratracheal instillation via a Penn Century Microsprayer and methacholine challenge via whole body inhalation. The intratracheal route was selected to maximize lung exposure to the test article/control solution. Whole body inhalation challenge has been selected for methacholine challenge in order to provoke an upper airway hypersensitivity response (i.e. bronchoconstriction).

Duration of treatment was one day.

Table 4 shows the experimental design. All animals were subjected to inhalation exposure of methacholine (500 µg/ml), 2 hours following TA/Control administration. All animals received a dose volume of 250 µl. Therefore, albuterol sulfate was diluted (in the control article and the 4 test articles) to concentrations of 0, 25, 50 and 100 µg/ml.

Thirty minutes prior to dosing, solutions of albuterol sulfate of 4 different concentrations (0, 25, 50 and 100 µg/ml) was made up in a 10x stock (500 µg/mL) in each of these four test article solutions (RDC1676-00, RDC1676-01, RDC1676-02; and RDC1676-03). These concentrations of albuterol sulfate were also made up in non-electrokinetically generated control fluid (control 1). The dosing solutions were prepared by making the appropriate dilution of each stock solution. All stock and dosing solutions were maintained on ice once prepared. The dosing was completed within one hour after the test/control articles are made. A solution of methacholine (500 µg/ml) was prepared on the day of dosing.

Each animal received an intratracheal instillation of test or control article using a Penn Century microsprayer. Animals were food deprived overnight and were anesthetized using isoflurane, the larynx was visualized with the aid of a laryngoscope (or suitable alternative) and the tip of the microsprayer was inserted into the trachea. A dose volume of 250 µl/animal of test article or control was administered.

The methacholine aerosol was generated into the air inlet of a mixing chamber using aeroneb ultrasonic nebulizers supplied with air from a Buxco bias flow pump. This mixing chamber in turn fed four individual whole body unrestrained plethysmographs, each operated under a slight negative pressure maintained by means of a gate valve located in the exhaust line. A vacuum pump was used to exhaust the inhalation chamber at the required flow rate.

Prior to the commencement of the main phase of the study, 12 spare animals were assigned to 3 groups (n=4/group) to determine the maximum exposure period at which animals may be exposed to methacholine to induce a severe but non-fatal acute bronchoconstriction. Four animals were exposed to methacholine (500µg/mL) for 30 seconds and respiratory parameters were measured for up to 10 minutes following commencement of aerosol. Methacholine nebulizer concentration and/or exposure time of aerosolization was adjusted appropriately to induce a severe but non-fatal acute/reversible bronchoconstriction, as characterized by an transient increase in penes.

Once prior to test article administration (Day -1) and again at 2, 6, 10, 14, 18, 22 and 26 hours postdose, animals were placed in the chamber and ventilatory parameters (tidal volume, respiratory rate, derived minute volume) and the enhanced pause Penh were measured for a period of 10 minutes using the Buxco Electronics BioSystem XA system, following commencement of aerosol challenge to methacholine. Once animals were within chambers baseline, values were recorded for 1-minute, following which methacholine, nebulizer concentration of 500ug/mL were aerosolized for 30 seconds, animals were exposed to the aerosol for further 10 minutes during which time ventilatory paramaters were continuously assessed . Penh was used as the indicator of bronchoconstriction; Penh is a derived value obtained from peak inspiratory flow, peak expiratory flow and time of expiration.
$$\text{Penh} = (\text{Peak expiratory flow} / \text{Peak inspiratory flow}) * (\text{Expiratory time} / \text{time to expire 65\% of expiratory volume} - 1).$$

Animals that did not display a severe acute broncoconstriction during the predose methacholine challenge were replaced. Any animal displaying at least 75% of their baseline PenhPenes value at 2 hours post dose were not included in the data analysis. The respiratory parameters were recorded as 20 second means.

Data considered unphysiological was excluded from further analysis.

Changes in Penh were plotted over a 15 minute period and Penh value was expressed as area under the curve. Numerical data was subjected to calculation of group mean values and standard deviations (as applicable).

Table 4. Experimental design; 7 male guinea pigs per group.

Group ID	Albuterol (0 µg/animal)	Albuterol (6/25 µg/animal)	Albuterol (12.5 µg/animal)	Albuterol (25 µg/animal)
1 (control 1) (ambient oxygen)	7 males	7 males	7 males	7 males
5 (RDC1676-00 (Solas)	7 males	7 males	7 males	7 males
6 (RDC1676-01 (20 ppm oxygen)	7 males	7 males	7 males	7 males
7 (RDC1676-02 (40 ppm oxygen)	7 males	7 males	7 males	7 males
8 (RDC1676-03 (60 ppm oxygen)	7 males	7 males	7 males	7 males

Results:

As shown in Figure 107A-D, in the absence of Albuterol, administration of the inventive electrokinetically generated fluids had no apparent effect on mean percent baseline PenH values, when measured over a 26 hour period.

Surprisingly, however, as shown in Figure 108A-D, administration of albuterol (representative data for the 25 µg albuterol/animal groups are shown) formulated in the inventive electrokinetically generated fluids (at all oxygen level values tested; ambient (Figure 108-A), 20 ppm (Figure 108-B), 40 ppm (Figure 108-C) and 60 ppm (Figure 108-D)) resulted in a striking prolongation of anti-bronchoconstrictive effects of albuterol, compared to control fluid. That is, the methacholine results showed a prolongation of the bronchodilation of albuterol out to at least 26 hours. Figures 108 A-D shows that there were consistent differences at all oxygen levels between RDC1676 and the normal saline control. Combining all 4 RDC1676 fluids, the p value for the overall treatment difference from normal saline was 0.03.

According to particular aspects of the present invention, therefore, the inventive electrokinetically generated solutions provide for synergistic prolongation effects with

Albuterol, thus providing for a decrease in a patient's albuterol usage, enabling more efficient cost-effective drug use, fewer side effects, and increasing the period over which a patient may be treated and responsive to treatment with albuterol.

EXAMPLE 4

Cytokine Profile

Mixed lymphocytes were obtained from a single healthy human volunteer donor. Buffy coat samples were washed according to standard procedures to remove platelets. Lymphocytes were plated at a concentration of 2×10^6 per plate in RPMI media (+ 50 mm HEPES) diluted with either inventive gas-enriched fluid or distilled water (control). Cells were stimulated with 1 microgram/mL T3 antigen, or 1 microgram/mL phytohemagglutinin (PHA) lectin (pan-T cell activator), or unstimulated (negative control). Following 24 hour incubation, cells were checked for viability and the supernatants were extracted and frozen.

The supernatants were thawed, centrifuged, and tested for cytokine expression using a XMAP® (Luminex) bead lite protocol and platform. Notably, IFN-gamma level was higher in the inventive gas-enriched culture media with T3 antigen than in the control culture media with T3 antigen, while IL-8 was lower in the inventive gas-enriched culture media with T3 antigen than in the control culture media with T3 antigen. Additionally, IL-6, IL-8, and TNF-alpha levels were lower in the inventive gas-enriched media with PHA, than in the control media with PHA, while IL-1b levels were lower in the inventive gas-enriched fluid with PHA when compared with control media with PHA. In gas-inventive media alone, IFN-gamma levels were higher than in control media.

Two million cells were plated into 6 wells of a 24-well plate in full RPMI + 50 mm Hepes with either inventive oxygen-enriched fluid (water) (wells 1, 3, and 5) or distilled water (2, 4 and 6) (10X RPMI diluted into water to make 1 x). Cells were stimulated with 1 ug/ml T3 antigen (wells 1 and 2) or PHA (wells 3 and 4). Control wells 5 and 6 were not stimulated. After 24 hours, cells were checked for viability and supernatants were collected and frozen. Next, the supernatants were thawed and spun at 8,000g to pellet. The clarified supernatants were assayed for the cytokines listed using a LUMINEX BEAD LITE protocol and platform. The numerical data is tabulated in Table 1.

TABLE 1

Sample	IFN	IL-10	IL-12p40	IL-12p70	IL-2	IL-4	IL-5	IL-6	IL-8	IL-1 β	IL-10	TNF α
1	0	0	0	2.85	0	0	7.98	20.3	1350	7.56	11500	15.5
2	0	0	0	3.08	0	0	8	15.2	8940	3.68	4280	7.94
3	0	581	168	3.15	0	0	8	16400	2200	3280	862	13700
4	0	377	56.3	4.22	0	0	8.08	23800	22100	33600	558	16200
5	0	0	0	2.51	0	0	7.99	24	1330	7.33	5900	8.55
6	0	0	0	2.77	0	0	8	5.98	3210	4.68	3330	0

EXAMPLE 5

Cytokine Expression

In particular aspects, human mixed lymphocytes were stimulated with T3 antigen or PHA in electrokinetically-generated oxygen-enriched fluid, or control fluid, and changes in IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, IFN- γ , GM-CSF, MIP-1 β , MCP-1, G-CSF, FGFb, VEGF, TNF- α , RANTES, Leptin, TNF- β , TFG- β , and NGF were evaluated. As can be seen from Figure 38, pro-inflammatory cytokines (IL-1 β , TNF- α , IL-6, and GM-CSF), chemokines (IL-8, MIP-1a, RANTES, and Eotaxin), inflammatory enzymes (iNOS, COX-2, and MMP-9), allergen responses (MHC class II, CD23, B7-1, and B7-2), and Th2 cytokines (IL-4, IL-13, and IL-5) tested were reduced in test fluid versus control fluid. By contrast, anti-inflammatory cytokines (e.g., IL1R-a, TIMPs) tested were increased in test fluid versus control fluid.

To expand on these data, Applicants used an art recognized model system involving ovalbumin sensitization, for assessing allergic hypersensitivity reactions. The end points studied were particular cytologic and cellular components of the reaction as well as serologic measurements of protein and LDH. Cytokine analysis was performed, including analysis of Eotaxin, IL-1A, IL-1B, KC, MCP-1, MCP-3, MIP-1A, RANTES, TNF-A, and VCAM.

Briefly, male Brown Norway rats were injected intraperitoneally with 0.5 mL Ovalbumin (OVA) Grade V (A5503-1G, Sigma) in solution (2.0 mg/mL) containing aluminum hydroxide (Al (OH)₃) (200 mg/mL) once each on days 1, 2, and 3. The study was a randomized 2 x 2 factorial arrangement of treatments (4 groups). After a two week waiting period to allow for an immune reaction to occur, the rats were either

exposed or were treated for a week with either RDC1676-00 (sterile saline processed through the Mixing Device), and RDC1676-01 (sterile saline processed through the Mixing Device with additional oxygen added). At the end of the 1 week of treatment for once a day, the 2 groups were broken in half and 50% of the rats in each group received either Saline or OVA challenge by inhalation._

Specifically, fourteen days following the initial serialization, 12 rats were exposed to RDC 1676-00 by inhalation for 30 minutes each day for 7 consecutive days. The air flow rate through the system was set at 10 liters/minute. A total of 12 rats were aligned in the pie chamber, with a single port for nebulized material to enter and evenly distribute to the 12 sub-chambers of the Aeroneb.

Fifteen days following initial sensitization, 12 rats were exposed to RDC 1676-01 by ultrasonic nebulization for 30 minutes each day for 7 consecutive days. The air flow was also set for 10 liters/minute, using the same nebulizer and chamber. The RDC 1676-00 was nebulized first and the Aeroneb chamber thoroughly dried before RDC 1676-01 was nebulized.

Approximately 2 hours after the last nebulization treatment, 6 rats from the RDC 1676-00 group were re-challenged with OVA (1% in saline) delivered by intratracheal instillation using a Penn Century Microsprayer (Model 1A-1B). The other 6 rats from the RDC 1676-00 group were challenged with saline as the control group delivered by way of intratracheal instillation. The following day, the procedure was repeated with the RDC 1676-01 group.

Twenty four hours after re-challenge, all rats in each group were euthanized by overdose with sodium pentobarbital. Whole blood samples were collected from the inferior vena-cava and placed into two disparate blood collection tubes: Qiagen PAXgene™ Blood RNA Tube and Qiagen PAXgene™ Blood DNA Tube. Lung organs were processed to obtain bronchoalveolar lavage (BAL) fluid and lung tissue for RT-PCR to assess changes in markers of cytokine expression known to be associated with lung inflammation in this model. A unilateral lavage technique was employed in order to preserve the integrity of the 4 lobes on the right side of the lung. The left "large" lobe was lavaged, while the 4 right lobes were tied off and immediately placed in TRIzol™, homogenized, and sent to the lab for further processing.

BAL analysis. Lung lavage was collected and centrifuged for 10 minutes at 4°C at 600-800 g to pellet the cells. The supernatants were transferred to fresh tubes and frozen at -80°C. Bronchial lavage fluid ("BAL") was separated into two aliquots. The

first aliquot was spun down, and the supernatant was snap frozen on crushed dry ice, placed in -80°C , and shipped to the laboratory for further processing. The amount of protein and LDH present indicates the level of blood serum protein (the protein is a serum component that leaks through the membranes when it's challenged as in this experiment) and cell death, respectively. The proprietary test side showed slight less protein than the control.

The second aliquot of bronchial lavage fluid was evaluated for total protein and LDH content, as well as subjected to cytological examination. The treated group showed total cells to be greater than the saline control group. Further, there was an increase in eosinophils in the treated group versus the control group. There were also slightly different polymorphonuclear cells for the treated versus the control side.

Blood analysis. Whole blood was analyzed by transfer of 1.2-2.0 mL blood into a tube, and allowing it to clot for at least 30 minutes. The remaining blood sample (approximately 3.5-5.0 mL) was saved for RNA extraction using TRIzolTM or PAXgeneTM. Next, the clotted blood sample was centrifuged for 10 minutes at 1200 g at room temperature. The serum (supernatant) was removed and placed into two fresh tubes, and the serum was stored at -80°C .

For RNA extraction utilizing Tri-Reagent (TB-126, Molecular Research Center, Inc.), 0.2 mL of whole blood or plasma was added to 0.75 mL of TRI Reagent BD supplemented with 20 μL of 5N acetic acid per 0.2 mL of whole blood or plasma. Tubes were shaken and stored at -80°C . Utilizing PAXgeneTM, tubes were incubated for approximately two hours at room temperature. Tubes were then placed on their side and stored in the -20°C freezer for 24 hours, and then transferred to -80°C for long term storage.

Luminex analysis. By Luminex platform, a microbead analysis was utilized as a substrate for an antibody-related binding reaction which is read out in luminosity units and can be compared with quantified standards. Each blood sample was run as 2 samples concurrently. The units of measurement are luminosity units and the groups are divided up into OVA challenged controls, OVA challenged treatment, and saline challenged treatment with proprietary fluid.

For Agilent gene array data generation, lung tissue was isolated and submerged in TRI Reagent (TR118, Molecular Research Center, Inc.). Briefly, approximately 1 mL of TRI Reagent was added to 50-100 mg of tissue in each tube. The samples were

homogenized in TRI Reagent, using glass-Teflon™ or Polytron™ homogenizer. Samples were stored at -80°C.

Blood Samples:

Figures 49-58 show the results of whole blood sample evaluations.

Exemplary Figure 49 shows the basic luminosity data presentation format for the blood sample data. Letters designating the identity of the measured cytokine (in this case KC) are at the top right of each data figure. The data is presented both as data points (upper graph) and bar graphs (lower graph) of the individual samples. In either case, the graphs are divided, from left to right, in four groups. The first 2 groups (RDC1676-00 OVA and RDC1676-01 OVA, respectively) were those that were re-challenged with OVA by inhalation, whereas the last two groups (RDC1676-00 OVA and RDC1676-01 OVA, respectively) were those that were re-challenged with saline control only. Again, the suffix 00 represents saline treatment and suffix 01 represents electrokinetically-generated fluid-treated groups.

Each blood sample was split into 2 samples and the samples were run concurrently. The units of measure are units of luminosity and the groups, going from left to right are: OVA challenged controls; OVA challenged electrokinetically-generated fluid treatment; followed by saline challenged saline treatment; and saline challenged electrokinetically-generated fluid treatment. To facilitate review, both the RDC1676-01 groups are highlighted with gray shaded backdrops, whereas the control saline treatment groups have unshaded backdrops.

Generally, in comparing the two left groups, while the spread of the RDC1676-01 group data is somewhat greater, particular cytokine levels in the RDC1676-01 group as a whole are less than the samples in the control treated group; typically about a 30% numerical difference between the 2 groups. Generally, in comparing the right-most two groups, the RDC1676-01 group has a slightly higher numerical number compared to the RDC1676-00 group.

Figure 50 shows analysis of RANTES (IL-8 super family) in blood sample data according to particular exemplary aspects. Luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion which again shows a 30-35% differential between the two groups, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Figure 51 shows analysis of MCP-1 in blood sample data according to particular exemplary aspects. Luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Figure 52 shows analysis of TNF alpha in blood sample data according to particular exemplary aspects. Luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Figure 53 shows analysis of MIP-1 alpha in blood sample data according to particular exemplary aspects. Luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Figure 54 shows analysis of IL-1 alpha in blood sample data according to particular exemplary aspects. Luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Figure 55 shows analysis of Vcam in blood sample data according to particular exemplary aspects. Luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Figure 56 shows analysis of IL-1 beta in blood sample data according to particular exemplary aspects. Luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Figures 57 and 58 show analysis of Eotaxin and MCP-3, respectively, in blood sample data according to particular exemplary aspects. In each case, luminosity units for the leftmost two groups (the OVA challenged groups) indicate that generally values in the RDC1676-01 treated group were less than the RDC1676-00 control group as shown by the dot plot in the upper graph portion, whereas in the saline only exposed groups the cytokine level values were roughly the same, or perhaps slightly increased in the RDC1676-01 treated group.

Bronchial Lavage Samples:

Figures 59-68 show the corresponding results of bronchoalveolar lavage fluid (BAL) sample evaluations.

Figure 59 shows analysis of KC in BAL data according to particular exemplary aspects. In this instance the response level, coupled with sampling variability, was inconclusive with respect to a difference between the RDC1676-01 and RDC1676-00-treated groups; that is, KC showed relatively little difference between the 2 groups, but the units of luminosity were very small.

Likewise, Figure 60 shows analysis of RANTES in BAL data according to particular exemplary aspects, and showing marked variability in the RDC1676-01 group with one reading being markedly higher than the others, skewing the results.

Likewise, Figure 61 shows analysis of TNF alpha in BAL data according to particular exemplary aspects, and showing relatively little significance in the way of difference between the RDC1676-01 and RDC1676-00-treated groups.

Figure 62 shows analysis of MCP-1 in BAL data according to particular exemplary aspects, and showing relatively little significance in the way of difference between the RDC1676-01 and RDC1676-00-treated groups.

Figures 63 through 68 show analysis of MIP1-A, IL-1 alpha, Vcam, IL-1 beta, MCP-3, and Eotaxin, respectively, in BAL data according to particular exemplary

aspects, and showing relatively little significance in the way of difference between the RDC1676-01 and RDC1676-00-treated groups.

In summary, this standard assay of inflammatory reaction to a known sensitization produced, at least in the blood samples, a marked clinical and serologic affect. Additionally, while significant numbers of control animals were physiologically stressed and nearly dying in the process, none of the RDC1676-01 treated group showed such clinical stress effects. This was reflected then in the circulating levels of cytokines, with approximately 30% differences between the RDC1676-01-treated and the RDC1676-01-treated groups in the OVA challenged groups. By contrast, there were small and fairly insignificant changes in cytokine, cellular and serologic profiles between the RDC1676-01-treated and the RDC1676-01-treated groups in the non-OVA challenged groups, which likely merely represent minimal baseline changes of the fluid itself.

EXAMPLE 6

Bradykinin B2 Receptor Affinity Binding

A Bio-Layer Interferometry biosensor, Octet Rapid Extended Detection (RED) (forteBio™) was utilized in order to examine membrane receptor affinity binding of Bradykinin ligand with the Bradykinin B2 receptor. The biosensor system consists of a polished fiber optic embedded into a polypropylene hub with a sensor-specific chemistry at the tip. The biosensor set-up has a layer of molecules attached to the tip of an optic fiber that creates an interference pattern at the detector. Any change in the number of molecules bound causes a measured shift in the pattern of light.

As shown in Figure 69 the Bradykinin B2 membrane receptor was immobilized onto aminopropylsilane (APS) biosensor. The sample plate set up was as designated in Figure 69 and analyzed in Figure 70. Next, the binding of Bradykinin to the immobilized receptor was assessed according to the sample set up as designated in Figure 71. Results of Bradykinin binding are shown in Figure 72. Bradykinin binding to the receptor was further titrated according to the set-up as designated in Figure 73.

As indicated in Figure 74, Bradykinin binding to the B2 receptor was concentration dependent, and binding affinity was increased in the proprietary gas-enriched saline fluid of the instant disclosure compared to normal saline. Stabilization of Bradykinin binding to the B2 receptor is shown in Figure 75.

EXAMPLE 7

(A regulatory T-cell assay was used to show effects of the inventive electrokinetically generated fluids in modulation of T-cell proliferation and elaboration of cytokines (Il-10) and other proteins (e.g., GITR, Granzyme A, XCL1, pStat, and Foxp3)) in regulatory T-cell assays, and of, for example, tryptase in PBMC)

The ability of particular embodiments disclosed herein to regulate T cells was studied by irradiating antigen presenting cells, and introducing antigen and T cells. Typically, these stimulated T cells proliferate. However, upon the introduction of regulatory T cells, the usual T cell proliferation is suppressed.

Methods:

Briefly, FITC-conjugated anti-CD25 (ACT-1) antibody used in sorting was purchased from DakoCytomation (Chicago, IL). The other antibodies used were as follows: CD3 (HIT3a for soluble conditions), GITR (PE conjugated), CD4 (Cy-5 and FITC-conjugated), CD25 (APC-conjugated), CD28 (CD28.2 clone), CD127-APC, Granzyme A (PE-conjugated), FoxP3 (BioLegend), Mouse IgG1 (isotype control), and XCL1 antibodies. All antibodies were used according to manufacturer's instructions.

CD4⁺ T cells were isolated from peripheral whole blood with CD4⁺ Rosette Kit (Stemcell Technologies). CD4⁺ T cells were incubated with anti-CD127-APC, anti-CD25-PE and anti-CD4-FITC antibodies. Cells were sorted by flow cytometry using a FACS Aria into CD4⁺CD25^{hi}CD127^{lo}/nTreg and CD4⁺CD25⁻ responder T cells.

Suppression assays were performed in round-bottom 96 well microtiter plates. 3.75×10^3 CD4⁺CD25^{neg} responder T cells, 3.75×10^3 autologous T reg, 3.75×10^4 allogeneic irradiated CD3-depleted PBMC were added as indicated. All wells were supplemented with anti-CD3 (clone HIT3a at 5.0 ug/ml). T cells were cultured for 7 days at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum. Sixteen hours before the end of the incubation, 1.0 mCi of ³H-thymidine was added to each well. Plates were harvested using a Tomtec cell harvester and ³H-thymidine incorporation determined using a Perkin Elmer scintillation counter. Antigen-presenting cells (APC) consisted of peripheral blood mononuclear cells (PBMC) depleted of T cells using StemSep human CD3⁺ T cell depletion (StemCell Technologies) followed by 40 Gy of irradiation.

Regulatory T cells were stimulated with anti-CD3 and anti-CD28 conditions and then stained with Live/Dead Red viability dye (Invitrogen), and surface markers CD4,

CD25, and CD127. Cells were fixed in the Lyze/Fix PhosFlow™ buffer and permeabilized in denaturing Permbuffer III®. Cells were then stained with antibodies against each particular selected molecule.

Statistical analysis was performed using the GraphPad Prism software. Comparisons between two groups were made by using the two-tailed, unpaired Student's t-test. Comparisons between three groups were made by using 1-way ANOVA. P values less than 0.05 were considered significant (two-tailed). Correlation between two groups were determined to be statistically significant via the Spearman coefficient if the r value was greater than 0.7 or less than -0.7 (two-tailed).

Results:

As indicated in Figure 76, regulatory T cell proliferation was studied by stimulating cells with diesel exhaust particulate matter (PM, from EPA). The x-axis of Figure 76 shows activated autologous CD4+ effector T cells (responder cells) as a solid black bar, and regulatory T cells alone in the gray bar (shown for confirmation of anergy) which were mixed at a 1:1 ratio as shown in the white bar. The y axis shows proliferation as measured by uptake of ³H-thymidine. As shown from left to right along the x-axis, "PM" indicates diesel exhaust derived Particulate Matter, "PM + Rev" indicates PM plus a gas-enriched electrokinetically generated fluid (Rev) of the instant disclosure, "Solis" indicates an electrokinetically generated fluid of the instant disclosure and device that is not gas-enriched beyond ambient atmosphere, only (no PM added), "Rev" indicates Rev alone (no PM added) as defined above, "Media" indicates the cell growth media alone control (minus PM; no Rev, no Solis), and "Saline Con" indicates the saline control (minus PM; no Rev, no Solis), "V" indicates verapamil, and "P" indicates propranolol, and "DT" is DT390 at 1:50.

As shown in Figure 77, cells stimulated with PM (no Rev, no Solis) resulted in a decrease in secreted IL-10, while cells exposed to PM in the presence of the fluids of the instant disclosure ("PM + Rev") resulted in a maintained or only slightly decreased production of IL-10 relative to the Saline and Media controls (no PM). Furthermore, Diphtheria toxin (DT390, a truncated diphtheria toxin molecule; 1:50 dilution of std. commercial concentration) was titrated into inventive fluid samples, and blocked the Rev-mediated effect of increase in IL-10 in Figure 77. Note that treatment with Rev alone resulted in higher IL-10 levels relative to Saline and Media controls.

Likewise, similar results, shown in Figures 78-82, were obtained with GITR, Granzyme A, XCL1, pStat, and Foxp3, respectively. In Figures, "NSC" is the same as "Solis" (no PM).

Figure 83 shows AA PBMC data, obtained from an allergic asthma (AA) profile of peripheral blood mononuclear cells (PBMC) evaluating tryptase. The AA PBMC data was consistent with the above T-regulatory cell data, as cells stimulated with particulate matter (PM) showed high levels of tryptase, while cells treated with PM in the presence of the fluids of the instant disclosure ("PM + Rev") resulted in significantly lower tryptase levels similar to those of the Saline and Media controls. Consistent with the data from T-regulatory cells, exposure to DT390 blocked the Rev-mediated effect on tryptase levels, resulting in an elevated level of tryptase in the cells as was seen for PM alone (minus Rev, no Rev, no Solis). Note that treatment with Rev alone resulted in lower tryptase levels relative to Saline and Media controls.

In summary, the data of Figure 76, showing a decreased proliferation in the presence of PM and Rev relative to PM in control fluid (no Rev, no Solis), indicates that the inventive electrokinetically generated fluid Rev improved regulatory T-cell function as shown by relatively decreased proliferation in the assay. Moreover, the evidence of this Example and Figures 76-83, indicate that beta blockade, GPCR blockade and Ca channel blockade affects the activity of Revera on Treg function.

EXAMPLE 8

(Treatment of primary bronchial epithelial cells (BEC) with the inventive electrokinetically generated fluids resulted in reduced expression and/or activity of two key proteins of the airway inflammatory pathways, MMP9 and TSLP)

Overview. As shown in Example 6 above (e.g., Figure 75, showing Stabilization of Bradykinin binding to the B2 receptor using Bio-Layer Interferometry biosensor, Octet Rapid Extended Detection (RED) (forteBioTM)), Bradykinin binding to the B2 receptor was concentration dependent, and binding affinity was increased in the electrokinetically generated fluid (e.g., Rev; gas-enriched electrokinetically generated fluid) of the instant disclosure compared to normal saline. Additionally, as shown in Example 7 in the context of T-regulatory cells stimulated with diesel exhaust particulate matter (PM, standard commercial source), the data showed a decreased proliferation of T-regulatory cells in the presence of PM and Rev relative to PM in control fluid (no Rev,

no Solis) (Figure 76), indicating that the inventive electrokinetically generated fluid Rev improved regulatory T-cell function; e.g., as shown by relatively decreased proliferation in the assay. Moreover, exposure to the inventive fluids resulted in a maintained or only slightly decreased production of IL-10 relative to the Saline and Media controls (no PM). Likewise, in the context of the allergic asthma (AA) profiles of peripheral blood mononuclear cells (PBMC) stimulated with particulate matter (PM), the data showed that exposure to the fluids of the instant disclosure ("PM + Rev") resulted in significantly lower tryptase levels similar to those of the Saline and Media controls. Additionally, the Diphtheria toxin (DT390, a truncated diphtheria toxin molecule; 1:50 dilution of std. commercial concentration) effects shown in Example 7 and Figures 76-83, indicate that beta blockade, GPCR blockade and Ca channel blockade affects the activity of the electrokinetically generated fluids on Treg and PBMC function. Furthermore, the data of Example 8 shows that, according to additional aspects, upon exposure to the inventive fluids, tight junction related proteins were upregulated in lung tissue. Figures 85-89 show upregulation of the junction adhesion molecules JAM 2 and 3, GJA1, 3, 4 and 5 (junctional adherins), OCLN (occludin), claudins (e.g., CLDN 3, 5, 7, 8, 9, 10), TJP1 (tight junction protein 1), respectively. Furthermore, as shown in the patch clamp studies of Example 15, the inventive electrokinetically generated fluids (e.g., RNS-60) affect modulation of whole cell conductance (e.g., under hyperpolarizing conditions) in Bronchial Epithelial Cells (BEC; e. g., Calu-3), and according to additional aspects, modulation of whole cell conductance reflects modulation of ion channels.

In this Example, Applicants have extended these discoveries by conducting additional experiments to measure the effects of production of two key proteins of the airway inflammatory pathways. Specifically, MMP9 and TSLP were assayed in primary bronchial epithelial cells (BEC).

Materials and Methods:

Commercially available primary human bronchial epithelial cells (BEC) (HBEpC-c from Promocell, Germany) were used for these studies. Approximately 50,000 cells were plated in each well of a 12 well plate until they reached ~80% confluence. The cells were then treated for 6 hours with normal saline, control fluid Solas or the test fluid Revera 60 at a 1:10 dilution (100ul in 1ml of airway epithelial growth medium) along with the diesel exhaust particulate matter (DEP or PM) before being lifted for FACS

analysis, as described in Example 8 herein. Both MMP9 and TSLP receptor antibodies were obtained from BD Biosciences and used as per manufacturer's specifications.

Results:

In Figures 115 and 116, DEP represents cells exposed to diesel exhaust particulate matter (PM, standard commercial source) alone, "NS" represents cells exposed to normal saline alone, "DEP+NS" represent cells treated with particulate matter in the presence of normal saline, "Revera 60" refers to cells exposed only to the test material, "DEP + Revera 60" refer to cells treated with particulate matter in the presence of the test material Revera 60. In addition, "Solas" and "DEP + Solas" represents cells exposed to the control fluid Solas alone or in combination with the particulate matter, respectively.

Figure 115 shows that the test material Revera 60 reduces DEP induced TSLP receptor expression in bronchial epithelial cells (BEC) by approximately 90%. Solas resulted in a 55% reduction in TSLP receptor expression, while Normal saline failed to produce similar level of reduction in TSLP receptor expression (approximately 20% reduction). The effect of the inventive solution in reducing TSLP receptor expression is a significant discovery in view of recent findings showing that TSLP plays a pivotal role in the pathobiology of allergic asthma and local antibody mediated blockade of TSLP receptor function alleviated allergic disease (Liu, YJ, Thymic stromal lymphopoietin: Master switch for allergic inflammation, *J Exp Med* 203:269-273, 2006; Al-Shami et al., A role for TSLP in the development of inflammation in an asthma model, *J Exp Med* 202:829-839, 2005; and Shi et al., Local blockade of TSLP receptor alleviated allergic disease by regulating airway dendritic cells, *Clin Immunol.* 2008, Aug 29. (Epub ahead of print)).

Likewise, Figure 116 shows the effect of Revera 60, Solas and normal saline on the DEP-mediated increase in MMP 9. Specifically, Revera 60 inhibited the DEP-induced cell surface bound MMP9 levels in bronchial epithelial cells by approximately 80%, and Solas had an inhibitory effect of approximately 70%, whereas normal saline (NS) had a marginal effect of about 20% reduction. MMP-9 is one of the major proteinases involved in airway inflammation and bronchial remodeling in asthma. Recently, it has been demonstrated that the levels of MMP-9 are significantly increased in patients with stable asthma and even higher in acute asthmatic patients compared with healthy control subjects. MMP-9 plays a crucial role in the infiltration of airway

inflammatory cells and the induction of airway hyperresponsiveness indicating that MMP-9 may have an important role in inducing and maintaining asthma (Vignola et al., Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis, *Am J Respir Crit Care Med* 158:1945–1950, 1998; Hoshino et al., Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma, *J Allergy Clin Immunol* 104:356–363, 1999; Simpson et al., Differential proteolytic enzyme activity in eosinophilic and neutrophilic asthma, *Am J Respir Crit Care Med* 172:559–565, 2005; Lee et al., A murine model of toluene diisocyanate-induced asthma can be treated with matrix metalloproteinase inhibitor, *J Allergy Clin Immunol* 108:1021–1026, 2001; and Lee et al., Matrix metalloproteinase inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma, *J Allergy Clin Immunol* 2003;111:1278–1284).

According to additional aspects, therefore, the inventive electrokinetically generated fluids have substantial therapeutic utility for modulating (e.g., reducing) TSLP receptor expression and/or for inhibiting expression and/or activity of MMP-9, including, for example, for treatment of inflammation and asthma.

EXAMPLE 9

(The inventive electrokinetically generated fluids were shown to have a synergistic anti-inflammatory effect with Budesonide in an art-recognized animal model for allergic asthma)

This working Example describes experiments performed to assess the airway anti-inflammatory properties of the inventive electrokinetically generated fluids (e.g., RDC-1676-03) in a Brown Norway rat ovalbumin sensitization model. The Brown Norway rat is an art-recognized model for determining the effects of a test material on airway function and this strain has been widely used, for example, as a model of allergic asthma. Airway pathology and biochemical changes induced by ovalbumin sensitization in this model resemble those observed in man (Elwood et al., *J Allergy Clin Immunol* 88:951–60, 1991; Sirois & Bissonnette, *Clin Exp Immunol* 126:9–15, 2001). The inhaled route was selected to maximize lung exposure to the test material or the control solution. The ovalbumin-sensitized animals were treated with budesonide

alone or in combination with the test material RDC 1676-03 for 7 days prior to ovalbumin challenge. 6 and 24 hours following the challenge, total blood count and levels of several pro and anti-inflammatory cytokines as well as various respiratory parameters were measured to estimate any beneficial effect of administering the test material on various inflammatory parameters.

Materials and Methods:

Brown Norway rats of strain Bn/Crl were obtained from Charles River Kingston, weighing approximately 275 ± 50 g at the onset of the experiment. All animal studies were conducted with the approval by PCS-MTL Institutional Animal Care and Use Committee. During the study, the use and care of animals were conducted according to guidelines of the USA National Research Council as well as Canadian Council of Animal Care.

Sensitization. On day 1 of the experiment, animals (14 animals in each treatment group) were sensitized by administration of a 1 ml intraperitoneal injection of a freshly prepared solution of 2 mg ovalbumin/100mg Aluminum Hydroxide per 1 ml of 0.9% Sodium Chloride, followed by repeat injection on day 3.

Treatment. Fifteen days following the initial sensitization, animals were subjected to nebulized exposure to control (Normal saline) or test solutions (electrokinetically generated fluids RDC1676-00, RDC1676-02 and RDC-1676-03), either administered alone or in combination with Budesonide, once daily for 15 minutes for 7 consecutive days. Animals were dosed in a whole body chamber of approximately 20L, and test atmosphere was generated into the chamber air inlet using aeroneb ultrasonic nebulizers supplied with air from a Buxco bias flow pump. The airflow rate was set at 10 liters/min.

Ovalbumin challenge. On day 21, 2 hours following treatment with the test solutions, all animals were challenged with 1% ovalbumin nebulized solution for 15 minutes (in a whole body chamber at airflow 2L/min).

Sample collection. At time points of 6 and 24 hours after the ovalbumin challenge, blood samples were collected for total and differential blood cell counts as well as for measuring levels of various pro and anti-inflammatory cytokines. In addition, Immediately after and at 6 and 24 hours following ovalbumin challenge the enhanced pause Penh and tidal volume were measured for a period of 10 minutes using the Buxco Electronics BioSystem XA system.

Results:

Eosinophil Count: As expected, and shown in Figure 109, treatment with Budesonide ("NS + Budesonide 750 µg/Kg"; densely crosshatched bar graph) reduced the total eosinophil count in the challenged animals relative to treatment with the normal saline "NS" alone control (open bar graph). Additionally, while treatment with the inventive fluid "RDC1676-03" alone (lightly crosshatched bar graph) did not significantly reduce the eosinophil count, it nonetheless displayed a substantial synergy with Budesonide in reducing the eosinophil count ("RDC1676-03 + Budesonide 750 µg/Kg", solid dark bar graph). Similarly, in Figure 110, the Eosinophil % also reflected a similar trend. While RDC1676-03 (lightly crosshatched graph bar) or Budesonide 750 ug/kg (densely crosshatched bar graph) alone did not have a significant effect on Eosinophil % count in the challenged animals, the two in combination reduced the Eosinophil % significantly (solid dark bar graph).

Therefore, Figure 109 and 110 show, according to particular aspects of the present invention that the inventive electrokinetically generated fluids (e.g., RDC1676-03) were demonstrated to have a substantial synergistic utility in combination with Budesonide to significantly reduce eosinophil count ("Eosinophil %" and total count) in an art-recognized rat model for human allergic asthma.

Respiratory parameters:

Figures 111 A-C and 112 A-C demonstrate the observed effect of the test fluids on Penh and tidal volume as measured immediately, 6 and 24 hours after the ovalbumin challenge. Penh is a derived value obtained from peak inspiratory flow, peak expiratory flow and time of expiration and lowering of penh value reflects a favorable outcome for lung function.

$$\text{Penh} = (\text{Peak expiratory flow/Peak inspiratory flow}) * (\text{Expiratory time/time to expire 65\% of expiratory volume} - 1).$$

As evident from figures 111 A-C, treatment with Budesonide (at both 500 and 750 ug/kg) alone or in combination with any of the test fluids failed to significantly affect the Penh values immediately after the challenge. However, 6 hours after the challenge, animals treated with RDC1676-03 alone or in combination with Budesonide 500 or 750 ug/kg demonstrated a significant drop in Penh values. Although the extent of this drop was diminished by 24 hours post challenge, the trend of a synergistic effect of Budesonide and RDC fluid was still observed at this time point.

Tidal volume is the volume of air drawn into the lungs during inspiration from the end-expiratory position, which leaves the lungs passively during expiration in the course of quiet breathing. As shown in figure 112 A-C, animals treated with Budesonide alone showed no change in tidal volumes immediately after the challenge. However, RDC1676-03 alone had a significant stimulatory effect on tidal volume even at this early time point. And again, RDC1676-03 in combination with Budesonide (both 500 and 750 ug/kg) had an even more pronounced effect on Tidal volume measurements at this time point. Six hours after the challenge, RDC1676-03 alone was sufficient to cause a significant increase in tidal volume and addition of Budesonide to the treatment regimen either alone or in combination had no added effect on tidal volume. Any effect observed at these earlier time points were, however, lost by the 24 hours time point.

Taken together, these data demonstrate that RDC1676-03 alone or in combination with Budesonide provided significant relief to airway inflammation as evidenced by increase in tidal volume and decrease in Penh values at 6 hours post challenge.

Cytokine Analysis:

To analyze the mechanism of the effects seen on the above discussed physiological parameters, a number of pro as well as anti-inflammatory cytokines were measured in blood samples collected at 6 and 24 hours after the challenge, immediately following the physiological measurements.

Figures 113A and 113B clearly demonstrate that Rev 60 (or RDC1676-03) alone lowered the blood level of eotaxin significantly at both 6 and 24 hours post challenge. Budesonide 750ug/kg also reduced the blood eotaxin levels at both of these time points, while Budesonide 250 ug/kg only had a notable effect at the later time point. However, the test solution Rev 60 alone showed effects that are significantly more potent (in reducing blood eotaxin levels) than both concentrations of Budesonide, at both time points. Eotaxin is a small C-C chemokine known to accumulate in and attract eosinophils to asthmatic lungs and other tissues in allergic reactions (e.g., gut in Crohn's disease). Eotaxin binds to a G protein coupled receptor CCR3. CCR3 is expressed by a number of cell types such as Th2 lymphocytes, basophils and mast cells but expression of this receptor by Th2 lymphocyte is of particular interest as these cells regulate eosinophil recruitment. Several studies have demonstrated increased

production of eotaxin and CCR3 in asthmatic lung as well as establishing a link between these molecules and airway hyperresponsiveness (reviewed in Eotaxin and the attraction of eosinophils to the asthmatic lung, Dolores M Conroy and Timothy J Williams *Respiratory Research* 2001, 2:150-156). It is of particular interest to note that these studies completely agree with the results in Figures 109 and 110 on eosinophil counts.

Taken together these results strongly indicate that treatment with RDC1676-03 alone or in combination with Budesonide can significantly reduce eosinophil total count and % in blood 24 hours after the ovalbumin challenge. This correlates with a significant drop in eotaxin levels in blood observed as early as 6 hours post challenge.

Blood levels of two major key anti-inflammatory cytokines, IL10 and Interferron gamma are also significantly enhanced at 6 hours after challenge as a result of treatment with Rev 60 alone or in combination with Budesonide. Figures 113C and 113D show such effects on Interferron gamma and IL 10, respectively. It is evident from these figures that Rev 60 alone or Rev 60 in combination with Budesonide 250 ug/kg significantly increased the blood level of IL10 in the challenged animals up to 6 hrs post challenge. Similarly, Rev 60 alone or in combination with Budesonide 250 or 750 ug/kg significantly increased the blood level of IFN gamma at 6 hours post challenge. Increase in these anti-inflammatory cytokines may well explain, at least in part, the beneficial effects seen on physiological respiratory parameters seen 6 hours post challenge, . The effect on these cytokines was no longer observed at 24 hour post challenge (data not shown).

Rantes or CCL5 is a cytokine expressed by circulating T cells and is chemotactic for T cells, eosinophils and basophils and has an active role in recruiting leukocytes into inflammatory sites. Rantes also activates eosinophils to release, for example, eosinophilic cationic protein. It changes the density of eosinophils and makes them hypodense, which is thought to represent a state of generalized cell activation. It also is a potent activator of oxidative metabolism specific for eosinophils.

As shown in Figure 114, systemic levels of Rantes was reduced significantly at 6 hours, but not at 24 hours post challenge in animals treated with Rev 60 alone or in combination of Budesonide 250 or 750 ug/kg. Once again, there is a clear synergistic effect of Budesonide 750 ug/kg and Rev 60 that is noted in this set of data. A similar downward trend was observed for a number of other pro-inflammatory cytokines, such

as KC or IL8, MCP3, IL1b, GCSF, TGFb as well as NGF, observed either at 6 or at 24 hours post challenge, in animals treated with Rev60 alone or in combination with Budesonide.

EXAMPLE 10

(The inventive therapeutic fluids have substantial utility for modulating intercellular tight junctions)

According to particular aspects, the inventive diffuser processed therapeutic fluids have substantial utility for modulating intercellular tight junctions, including those relating with pulmonary and systemic delivery and bioavailability of polypeptides, including the exemplary polypeptide salmon calcitonin (sCT).

Example Overview. Salmon calcitonin (sCT) is a 32 amino acid peptide with a molecular weight of 3,432 Daltons. Pulmonary delivery of calcitonin has been extensively studied in model systems (e.g., rodent model systems, rat model systems, etc) to investigate methods to enhance pulmonary drug delivery (e.g., intratracheal drug delivery). According to particular exemplary aspects, the inventive diffuser processed therapeutic fluid has substantial utility for modulating (e.g., enhancing) intercellular tight junctions, for example those associated with pulmonary and systemic delivery and bioavailability of sCT in a rat model system.

Methods:

Intratracheal drug delivery. According to particular embodiments, sCT is formulated in the inventive therapeutic fluid and administered to rats using an intratracheal drug delivery device. In certain aspects, a Penn Century Micro-Sprayer device designed for rodent intratracheal drug delivery is used, allowing for good lung delivery, but, as appreciated in the art, with relatively low alveolar deposition resulting in poor systemic bioavailability of peptides. According to particular aspects, this art-recognized model system was used to confirm that the inventive diffuser processed therapeutic fluid has substantial utility for modulating (e.g., enhancing) intercellular tight junctions, including those associated with pulmonary and systemic delivery and bioavailability of polypeptides.

Animal groups and dosing. In certain aspects, rats are assigned to one of 3 groups (n=6 per group): a) sterile saline; b) base solution without O₂ enrichment ('base solution'); or c) inventive diffuser processed therapeutic fluid ('inventive enriched based

solution'). The inventive enriched based solution is formed, for example by infusing oxygen in 0.9% saline. Preferably, the base solution comprises about 0.9% saline to minimize the potential for hypo-osmotic disruption of epithelial cells. In certain embodiments, sCT is separately reconstituted in the base solution and the inventive enriched based solution and the respective solutions are delivered to respective animal groups by intratracheal instillation within 60 minutes (10 µg sCT in 200 µL per animal).

Assays. In particular aspects, blood samples (e.g., 200 µl) are collected and placed into EDTA coated tubes prior to dosing and at 5, 10, 20, 30, 60, 120 and 240 minutes following dosing. Plasma is harvested and stored at = -70°C until assayed for sCT using an ELISA.

For Agilent gene array data generation, lung tissue was isolated and submerged in TRI Reagent (TR118, Molecular Research Center, Inc.). Briefly, approximately 1 mL of TRI Reagent was added to 50-100 mg of tissue in each tube. The samples were homogenized in TRI Reagent, using glass-Teflon™ or Polytron™ homogenizer. Samples were stored at -80°C.

Results:

Enhancement of tight junctions. Figure 84 shows that RDC1676-01 (sterile saline processed through the instant proprietary device with additional oxygen added; gas-enriched electrokinetically generated fluid (Rev) of the instant disclosure) decreased systemic delivery and bioavailability of sCT. According to particular aspects, the decreased systemic delivery results from decreased adsorption of sCT, most likely resulting from enhancement of pulmonary tight junctions. RDC1676-00 signifies sterile saline processed according to the presently disclosed methods, but without oxygenation.

Additionally, according to particular aspects, tight junction related proteins were upregulated in lung tissue. Figures 85-89 show upregulation of the junction adhesion molecules JAM 2 and 3, GJA1,3,4 and 5 (junctional adherins), OCLN (occludin), claudins (e.g., CLDN 3, 5, 7, 8, 9, 10), TJP1 (tight junction protein 1), respectively.

EXAMPLE 11

(The inventive therapeutic fluids have substantial utility for modulating Nitric Oxide levels)

According to particular aspects, the inventive diffuser processed therapeutic fluids have substantial utility for modulating nitric oxide levels, and/or related enzymes. Figures 90-94 show data obtained from human foreskin keratinocytes exposed to

RDC1676-01 (sterile saline processed through the instant proprietary device with additional oxygen added; gas-enriched electrokinetically generated fluid (Rev) of the instant disclosure) showing up-regulation of NOS1 and 3, and Nostrin, NOS3. By contrast, data obtained from rat lung tissue (tissue of above Example entitled "Cytokine Expression") shows down regulation of NOS2 and 3, Nostrin and NOS1AP with Rev (Figures 93, 94).

EXAMPLE 12

(Localized electrokinetic effects (voltage/current) were demonstrated using a specially designed mixing device comprising insulated rotor and stator features)

In this Example, feature-localized electrokinetic effects (voltage/current) were demonstrated using a specially designed mixing device comprising insulated rotor and stator features.

Overview. As discussed in detail herein above under "Double Layer Effect" (see also Figures 26 and 28) The mixing device 100 may be configured to create the output material 102 by complex and non-linear fluid dynamic interaction of the first material 110 and the second material 120 with complex, dynamic turbulence providing complex mixing that further favors electrokinetic effects. According to particular aspects, the result of these electrokinetic effects may be present within the output material 102 as charge redistributions and redox reactions, including in the form of solublized electrons that are stabilized within the output material.

In addition to general surface-related double layer effects in the mixing chamber, Applicants additionally reasoned that localized electrokinetic effects may be imparted by virtue of the feature-induced microcavitation and fluid acceleration and deceleration in the vicinity of the features. The studies of this Example were thus performed to further investigate and confirm said additional electrokinetic aspects.

Materials:

A test device similar to the inventive mixing devices described herein was constructed, comprising a stainless steel rotor **12** having two features **18** (disposed at 180 degrees), and a stator **14** with a single feature **16** positioned to be rotationally opposable to the rotor features **18** and stator features **16**. Significantly, the rotor and stator features, in each case, are insulated from the respective rotor and stator bodies (Figure 95). The device was machined to provide for a consistent rotor:stator gap **20** of

0.020 inches to conform with the devices disclosed elsewhere herein. There is a rotating contact (not shown) at the end of the rotor shaft (not shown) that provides an electrical path for the rotor surface and for the insulated rotor features. Likewise the stator has a similar insulated feature **16** (Figure 95), wherein the stator inner surface and the insulated stainless steel feature are connected to respective contacts on the stator exterior.

A operational amplifier (OpAmp) circuit (M) **22** is connected between the contacts. The operational amplifier (OpAmp) circuit was constructed to provide for collection of very low voltage measurements by taking advantage of the high input impedance of such amplifiers. The outputs of the OpAmp are fed to the inputs of an oscilloscope (e.g., a battery powered laptop running an oscilloscope application with a Pico Scope 3000TM).

To eliminate the introduction of any ambient noise (e.g., RF radiation from wireless network signals and from the 60 Hz power line) during testing of the device, a fine copper mesh, RF-shielded compartment (approx. three by four by four feet) was constructed to provide a Faraday cage. This configuration provided for excellent signal to noise ratios during experimental testing, as interfering signals from 60 Hz AC noise (e.g., of approximately two volts) and high frequency RF was reduced well below the signals of interest. Using a battery powered laptop running an oscilloscope application with a Pico Scope 3000 enabled detection of the 30 mV signals (as in Figure 96) created by the features of the test device. In addition, a variable speed DC motor was positioned outside the Faraday cage and coupled to the rotatable test device via a non-metallic shaft to effectively isolate the motor noise away from the test device.

Methods:

The OpAmp circuit was used to measure voltage potential between the contacts connecting the stator inner surface **12** and the insulated stator feature **16**. With the particular circuit arrangement, only a potential was measured. The rotational speed of the device could be varied between about 700 to about 2800 rpm (with the data of Figure 96 being measured with the device running at about 1800 rpm).

To avoid any extraneous voltage generation due to a pump or peristaltic pump, fluid flow through the device was accomplished using inert nitrogen or air or argon acting on fluid in tanks connected to the device. There was no perceptible voltage

contribution from the flow mechanism, and typically air was used as the pumping force to provide for fluid flow through the device.

Fluid flow rate through the device was about 1 L/min.

An initial set of non-rotational experiments was conducted by directing fluid flow through the device chamber but without rotation of the rotor in order to assess the presence of any voltage between the stator body **12** and the isolated feature **16**. Separate experiments were conducted for both flow directions.

An additional set of rotational experiments was then conducted with the same fluid flow rate, and with the device rotor rotating at various speeds from about 300 to about 1800 rpm. For any given experiment, the flow rate and rotational speed were held constant.

Results:

With respect to the non-rotational experiments, with fluid flowing through the device in either direction without any rotor rotation there was only a barely perceptible voltage (e.g., 1 to 2 mV) between the body of the stator and the insulated feature.

With respect to the rotational experiments, and with reference to Figure 96, it can be seen that voltage pulses (potential pulses), temporally correlating (in this case at about 1800 rpm) with rotational alignment of opposing rotor stator features, were measurable with the OpAmp in the operating test device. Moreover, such periodic voltage pulses, correlating with feature alignments, could be observed over a range from about 250 or 300 rpm to about 1800. Additionally, with or without fluid flow, such voltage pulses were observed in the rotational experiments as long as the cavity/fluid chamber of the device was filled with fluid. According to particular aspects, and without being bound by mechanism, rapid, violent compression (e.g., cavitation), acceleration and deceleration of fluid flow in the vicinity of the repetitive rotationally aligned features created the respective local voltage pulses that correlate exactly with the rotational period, providing, at least in part, for electrokinetically generated fluid according to the present invention. Additional experiments revealed that the amplitude (peak shape and height) of the voltage pulses increased with increasing rotational velocity, being initially observable at about 250 to 300 rpm in this particular test device, and increasing up to at least about 2800 rpm. The magnitude of the violent acceleration and deceleration, etc., of fluid flow in the vicinity of the rotationally aligned features would be expected to generally increase with increasing rotational velocity; at least until a maximum was

reached reflecting physical limits imposed by the geometry, configuration and/or flow rate of the device. According to additional aspects, because localized voltage spikes are present, localized current flow (e.g., current pulses) is generated in the vicinity of the features, providing, at least in part, for electrokinetically generated fluid according to the present invention (e.g., without being bound by mechanism, providing for electrochemical reactions as discussed elsewhere herein).

According to additional aspects, and without being bound by mechanism, such feature-localized effects (e.g., voltage pulses and current and/or currents pulses) contribute to generation of the electrokinetically generated fluids in combination with more general surface-related double layer and streaming current effects discussed elsewhere herein above under "Double Layer Effect" (see also Figures 26 and 28).

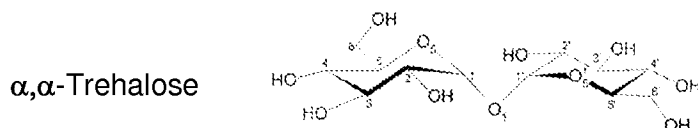
EXAMPLE 13

(Relative to non-electrokinetically generated control fluids, the inventive electrokinetically generated fluids were shown to differentially affect line widths in ^{13}C NMR analysis of the dissolved solute α,α -Trehalose)

Overview. Applicants data disclosed elsewhere herein support utility and mechanism wherein the inventive electrokinetically generated fluids mediate regulation or modulation of intracellular signal transduction by modulation of at least one of cellular membranes, membrane potential/conductance, membrane proteins (e.g., membrane receptors such as G protein coupled receptors), calcium dependant cellular signaling systems, and intercellular junctions (e.g., tight junctions, gap junctions, zona adherins and desmosomes). Specifically, using a variety of art-recognized biological test systems and assays, Applicants data shows, relative to control fluids, differential effects of the inventive fluid on, for example: regulatory T cell proliferation; cytokine and protein levels (e.g, IL-10, GITR, Granzyme A, XCL1, pStat5, and Foxp3, tyrptase, tight junction related proteins, TSLP receptor, MMP9, etc.); binding of Bradykinin ligand with the Bradykinin B2 receptor; expression of TSLP receptor, whole cell conductance; etc. Moreover, the Diphtheria toxin (DT390) effects shown herein indicate that beta blockade (beta 2 adrenergic receptor), and/or GPCR blockade and/or Ca channel blockade affects the activity of the electrokinetically generated fluids on, for example, Treg and PBMC function.

Taken together these effects indicate that the inventive electrokinetically generated fluids are not only fundamentally distinguished from prior art fluids, but also that they provide for novel compositions and substantial utilities such as those presently disclosed and claimed herein.

In this Example. Applicants have in this Example performed nuclear magnetic resonance (NMR) studies to further characterize the fundamental nature of the inventive electrokinetically generated fluids. Specifically, Applicants have analyzed the ^{13}C NMR spectra of α,α -Trehalose dissolved in the electrokinetically generated fluid, compared to dissolution in non-electrokinetically generated fluid. Trehalose (shown below with carbons numbered for reference) is a cosmotrophic solute and is known, for example to protect against protein denaturation, membrane desiccation, organism viability upon freezing, etc. Applicants, given the data summarized above, reasoned that α,α -Trehalose might provide an effective tool to further probe the properties/structure of the inventive electrokinetically generated fluids. Applicants reasoned that NMR-related ‘chemical shifts’ and effects on ‘line widths’ could be used to assess properties of the inventive fluids. For these studies, a non-superoxygenated inventive electrokinetically generated fluid (referred to herein as “Solas”) was employed to minimize the possibility that paramagnetic impurities, such as dissolved oxygen, might act to counter or otherwise mask the effects being analyzed.



Materials and Methods:

Solution Preparation. The Phosphate (sodium salt) and D-(+)-Trehalose dihydrate (T9531-10G, reduced metal content) and 99.9% D₂O containing 1% DSS were purchased from Sigma. The “Normal Saline” is 0.9% Sodium Chloride, pH 5.6 (4.5-7.0), from Hospira. The 0.25 M α,α -Trehalose solutions were prepared by dissolving 0.949 g trehalose into 965 μL Normal Saline and 35 mL Phosphate Buffered Saline (100mM Phosphate Buffer in 0.9% NaCl prepared in such a way that when 35 μL of this buffer are added to 1.0 mL trehalose solution the pH becomes 6.93).

Nuclear Magnetic Resonance Spectra Collection. Spectra were collected at the University of Washington NMR facility using either an 500 MHz or 300 MHz Bruker Avance series instrument fitted with a Bruker BBO : X {1H} probe and running XWINNMR 3.5. ^{13}C NMR spectra were collected at 125.7 MHz or 75.46 MHz using a 14000 Hz or 7900 Hz sweep width using 64K or 128K data points and 128 or 256 scans. The resulting FIDs were zero-filled twice and processed with a 1.0 Hz line broadening factor. Temperature was controlled using the Bruker Biospin Variable Temperature unit. External deuterium locking was employed by placing 99.9% D₂O + 1% DSS + a trace of acetone in a coaxial NMR insert tube, purchased from Wilmad. The NMR data was processed using the iNMR software v. 2.6.4 from Mestrelab Research.

Results:

Sample Spectra. FIGURE 97A-C shows expansions of six ^{13}C -NMR spectra overlaid on top of each other such that the DSS signals line up at -2.04 ppm. The DSS signals are shown at the far right of the figure, and the acetone methyl signal is shown near 30.9 ppm. The remaining signals correspond to the 6 carbons of trehalose as shown in the α,α -Trehalose structure above. As can be seen, the carbon signals in the Solas solutions show small chemical shifts (generally upfield) compared to the control solutions.

Line Width Measurements. TABLE 1 below shows the measured ^{13}C NMR line widths for the six carbons of trehalose and the methyl carbon of acetone at 3 different temperatures for Solas Saline (an inventive electrokinetically generated fluid). The corresponding Normal Saline samples represent non-electrokinetic control solutions at each temperature. In the Solas solutions, the line widths are significantly different from the line widths in the control solution for each carbon atom. The smaller linewidths in the Solas solutions at lower temperatures likely result from a faster tumbling rate of the trehalose molecule as a whole (including any solvated water molecules) compared to the control solutions.

TABLE 1. ^{13}C NMR Line Widths for α,α -Trehalose in Solas & Normal Saline^{a,b}

Test Fluid (Temp. degrees K)	C-1	C-2	C-3	C-4	C-5	C-6	Acetone
Solas (277)	8.4	8.22	8.3	8.15	8.3	11.1	5.1
Normal (269.9)	15.4	16.1	15.8	14.9	15.4	21.7	5.1

Solas (293)	9.52	8.7	9.28	9	8.9	11.25	5.63
Normal (292.9)	10.33	10.23	10.23	9.93	10.23	13.13	5.63
Solas (310)	2.28	2.03	2.18	2.19	2	2.55	0.67
Normal (309.9)	1.17	0.99	1.1	1.02	0.97	1.42	0.67

^a1.0 Hz was subtracted from all line width values due to the 1.0 Hz line broadening used during processing. In addition, line width values were normalized relative to the acetone signal in the external reference tube in order to compensate for magnetic field inhomogeneities. This was done by subtracting from the Normal Saline line widths the amount by which the acetone peak was broadened in the corresponding Solas Saline spectra.

^bError in line width measurements estimated to be within +/-0.30 Hz

The ¹³C NMR line widths for α,α -Trehalose in Solas and normal saline, in each case normalized with respect to the Acetone line, are shown graphically in Figure 97A. In conclusion, the NMR data for ¹³C NMR line widths for α,α -Trehalose in Solas and normal saline indicate that there is a property of the inventive solution which alters solute tumbling.

Taken together with the biological activities summarize above and elsewhere herein, these ¹³C NMR line width effects indicate that the inventive electrokinetically generated fluids are not only fundamentally distinguished from prior art fluids in terms of solute interactions, but also that they provide for novel compositions and substantial utilities such as those presently disclosed and claimed herein.

EXAMPLE 14

(Relative to non-electrokinetically generated control fluids, the inventive electrokinetically generated fluids produced differential square wave voltametry profiles and displayed unique electrochemical properties under stripping polarography)

Overview. Applicants' data disclosed elsewhere herein support utility and mechanism wherein the inventive electrokinetically generated fluids mediate regulation or modulation of intracellular signal transduction by modulation of at least one of cellular membranes, membrane potential/conductance, membrane proteins (e.g., membrane receptors such as G protein coupled receptors), calcium dependant cellular signaling systems, and intercellular junctions (e.g., tight junctions, gap junctions, zona adherins and desmosomes). Specifically, using a variety of art-recognized biological test systems and assays. Applicants data shows, relative to control fluids, differential effects of the inventive fluid on, for example: regulatory T cell proliferation; cytokine and protein

levels (e.g, IL-10, GTR, Granzyme A, XCL1, pStat5, and Foxp3, typtase, tight junction related proteins, TSLP receptor, MMP9, etc.); binding of Bradykinin ligand with the Bradykinin B2 receptor; expression of TSLP receptor, whole cell conductance; etc. Moreover, the Diphtheria toxin (DT390) effects shown herein indicate that beta blockade (beta 2 adrenergic receptor), and/or GPCR blockade and/or Ca channel blockade affects the activity of the electrokinetically generated fluids on, for example, Treg and PBMC function.

Taken together these effects indicate that the inventive electrokinetically generated fluids are not only fundamentally distinguished from prior art fluids, but also that they provide for novel compositions and substantial utilities such as those presently disclosed and claimed herein.

In this Example. Applicants have, in this Example, performed voltametry studies to further characterize the fundamental nature of the inventive electrokinetically generated fluids. Voltametry is frequently used to determine the redox potential or measure kinetic rates and constants of fluids. The common characteristic of all voltametric methods is that they involve the application of a potential to an electrode and the resultant current flowing is monitored through an electrochemical cell. The applied potential produces a change in the concentration of an electroactive species at the electrode surface by electrochemically reducing or oxidizing the species.

Specifically, Applicants have utilized voltametric methods (i.e., square wave voltametry and stripping polarography) to further characterize fundamental differences between control saline fluid and the inventive electrokinetically generated test fluids (e.g., Solas and Revera). Applicants, given the biological and membrane effects data summarized above, reasoned that square wave voltametry and stripping polarography would provide an effective means to further characterize the unique properties of the inventive electrokinetically generated fluids.

Applicants further reasoned that differences in current at specific voltages, production of different concentrations of an electroactive redox compound, creation of new redox compounds, and possession of unique electrochemical properties could be used to assess and characterize properties of the inventive fluids. For these studies, both a superoxygenated electrokinetically generated fluid (Revera), and a non-superoxygenated inventive electrokinetically generated fluid (Solas) were used.

Materials and Methods:

Materials and Solution Preparation. The experiments were conducted on an EG & G SMDE 303A polarograph (Princeton Applied Research). The electrolyte, NaOH, used in the square wave voltammetry experiment, was purchased from Sigma. A 10 mL sample of the inventive fluid solution was prepared by adding 100 μ L of NaOH to 9.9 mL of Revera Saline to make a 0.18 molar solution. With regards to the stripping polarography experiment, no extra electrolyte was utilized.

Square Wave Voltammetry. As stated above, voltammetry is used to determine the redox potential or measure kinetic rates and constants in fluids. In the square wave voltammetry experiment, a potential of 0.0 to approximately -1.75 V was applied to an electrode and the resultant current flowing through the electrochemical cell was monitored.

Stripping Polarography. The stripping polarography method is similar to the square wave voltammetry method. However, no electrolyte was utilized as stated above and also involved a pre-step. In the pre-step, the static mercury drop electrode was held for 30 seconds at -1.1 V to amalgamate any compounds whose reduced form was soluble in mercury. Then, the potentials between -1.1 V and 0.0 V were scanned and the resultant current flowing through the electrochemical cell was monitored. A linear scan into the negative potentials on this amalgam provided a sensitive measurement of these compounds.

Results:

Square Wave Voltammetry. As evident from figure 98, the current profiles at -0.14V, -0.47V, -1.02V and -1.36V differ between the various tested agents. According to particular aspects, the differences in current generated at the various specific voltages indicate at least one of a different concentration of an electroactive redox compound and/or a new or unique electroactive redox compound, and/or a change in the diffusion-limiting electrical double layer surrounding the mercury drop.

Stripping Polarography. Figure 99 shows that the inventive electrokinetically generated fluids, Revera and Solas, show unique spectra with pronounced peaks at -0.9 volts that are not present in the non-electrokinetically generated blank and saline control fluids. Additionally, the spectra of the non-electrokinetically generated blank and saline control fluids show characteristic peaks at -0.19 and -0.3 volts that are absent in the spectra for the electrokinetically generated Solas and Revera fluids.

According to particular aspects, therefore, these results show unique electrochemical properties of the inventive electrokinetically generated Solas and Revera fluids compared to non-electrokinetically generated Saline control fluid. According to additional aspects, the results indicate the presence or generation of at least one of a different concentration of an electroactive redox compound and a new and/or unique electroactive redox compound in electrokinetically generated versus non-electrokinetically generated fluids.

On top of the various biological data presented elsewhere herein, this differential voltametry data, particularly when considered along with the differential effects on whole cell conductance, ^{13}C NMR line-width analysis, and the mixing device feature-localized effects (e.g., voltage pulses and current and/or currents pulses) indicate that the inventive electrokinetically generated fluids are not only fundamentally distinguished from prior art fluids, but also provide for novel compositions and substantial utilities such as those presently disclosed and claimed herein.

EXAMPLE 15

(Patch clamp analysis conducted on bronchial epithelial cells (BEC) perfused with inventive electrokinetically generated fluid (RNS-60) revealed that exposure to RNS-60 resulted in a decrease in whole cell conductance, and stimulation with a cAMP stimulating "cocktail", which dramatically increased the whole-cell conductance. also increased the drug-sensitive portion of the whole-cell conductance, which was ten-times higher than that observed under basal conditions)

In this Example, patch clamp studies were performed to further confirm the utility of the inventive electrokinetically generated fluids to modulate intracellular signal transduction by modulation of at least one of membrane structure, membrane potential or membrane conductivity, membrane proteins or receptors, ion channels, and calcium dependant cellular messaging systems.

Overview. As shown in Example 6 above (e.g., Figure 75, showing Stabilization of Bradykinin binding to the B2 receptor using Bio-Layer Interferometry biosensor, Octet Rapid Extended Detection (RED) (forteBioTM)), Bradykinin binding to the B2 receptor was concentration dependent, and binding affinity was increased in the electrokinetically generated fluid (e.g., Rev; gas-enriched electrokinetically generated fluid) of the instant disclosure compared to normal saline. Additionally, as shown in

Example 7 in the context of T-regulatory cells stimulated with particulate matter (PM), the data showed a decreased proliferation of T-regulatory cells in the presence of PM and Rev relative to PM in control fluid (no Rev, no Solis) (Figure 76), indicating that the inventive electrokinetically generated fluid Rev improved regulatory T-cell function; e.g., as shown by relatively decreased proliferation in the assay. Moreover, exposure to the inventive fluids resulted in a maintained or only slightly decreased production of IL-10 relative to the Saline and Media controls (no PM). Likewise, in the context of the allergic asthma (AA) profiles of peripheral blood mononuclear cells (PBMC) stimulated with particulate matter (PM), the data showed that exposure to the fluids of the instant disclosure ("PM + Rev") resulted in significantly lower tryptase levels similar to those of the Saline and Media controls. Additionally, the Diphtheria toxin (DT390) effects shown in Example 7 and figures 76-83, indicate that beta blockade, GPCR blockade and Ca channel blockade affects the activity of the electrokinetically generated fluids on Treg and PBMC function. Furthermore, the data of Example 8 shows that, according to additional aspects, upon expose to the inventive fluids, tight junction related proteins were upregulated in lung tissue. Figures 85-89 show upregulation of the junction adhesion molecules JAM 2 and 3, GJA1,3,4 and 5 (junctional adherins), OCLN (occludin), claudins (e.g., CLDN 3, 5, 7, 8, 9, 10), TJP1 (tight junction protein 1), respectively.

Patch clamp studies were performed to further investigate and confirm said utilities.

Materials and Methods:

The Bronchial Epithelial line Calu-3 was used in Patch clamp studies. Calu-3 Bronchial Epithelial cells (ATCC #HTB-55) were grown in a 1:1 mixture of Ham's F12 and DMEM medium that was supplemented with 10% FBS onto glass coverslips until the time of the experiments. In brief, a whole cell voltage clamp device was used to measure effects on Calu-3 cells exposed to the inventive electrokinetically generated fluids (e.g., RNS-60; electrokinetically treated normal saline comprising 60 ppm dissolved oxygen; sometimes referred to as "drug" in this Example).

Patch clamping techniques were utilized to assess the effects of the test material (RNS-60) on epithelial cell membrane polarity and ion channel activity. Specifically, whole cell voltage clamp was performed upon the Bronchial Epithelial line Calu-3 in a bathing solution consisting of: 135mM NaCl, 5mM KCl, 1.2mM CaCl₂, 0.8mM MgCl₂,

and 10mM HEPES (pH adjusted to 7.4 with N-methyl D-Glucamine). Basal currents were measured after which RNS-60 was perfused onto the cells.

More specifically, patch pipettes were pulled from borosilicate glass (Garner Glass Co, Claremont, CA) with a two-stage Narishige PB-7 vertical puller and then fire-polished to a resistance between 6-12 Mohms with a Narishige MF-9 microforge (Narishige International USA, East Meadow, NY). The pipettes were filled with an intracellular solution containing (in mM): 135 KCl, 10 NaCl, 5 EGTA, 10 Hepes, pH was adjusted to 7.4 with NMDG (N-Methyl-D-Glucamine).

The cultured Calu-3 cells were placed in a chamber containing the following extracellular solution (in mM): 135 NaCl, 5 KCl, 1.2 CaCl₂, 0.5 MgCl₂ and 10 Hepes (free acid), pH was adjusted to 7.4 with NMDG.

Cells were viewed using the 40X DIC objective of an Olympus IX71 microscope (Olympus Inc., Tokyo, Japan). After a cell-attached gigaseal was established, a gentle suction was applied to break in, and to attain the whole-cell configuration. Immediately upon breaking in, the cell was voltage clamped at -120, -60, -40 and 0 mV, and was stimulated with voltage steps between ± 100 mV (500 ms/step). After collecting the whole-cell currents at the control condition, the same cell was perfused through bath with the test fluid comprising same extracellular solutes and pH as for the above control fluid, and whole-cell currents at different holding potentials were recorded with the same protocols.

Electrophysiological data were acquired with an Axon Patch 200B amplifier, low-pass filtered at 10 kHz, and digitized with 1400A Digidata (Axon Instruments, Union City, CA). The pCLAMP 10.0 software (Axon Instruments) was used to acquire and to analyze the data. Current (I)-to-voltage (V)

relationships (whole cell conductance) were obtained by plotting the actual current value at approximately 400 msec into the step, versus the holding potential (V). The slope of the I/V relationship is the whole cell conductance.

Drugs and Chemicals. Whenever indicated, cells were stimulated with a cAMP stimulatory cocktail containing 8-Br-cAMP (500 mM), IBMX (isobutyl-1-methylxanthine, 200 mM) and forskolin (10 mM). The cAMP analog 8-Br-cAMP (Sigma Chem. Co.) was used from a 25 mM stock in H₂O solution. Forskolin (Sigma) and IBMX (Sigma) were used from a DMSO solution containing both 10 mM Forskolin and 200 mM IBMX stock solution.

Patch Clamp Results:

Figure 100 shows whole-cell currents under basal (no cAMP) conditions, with a protocol stepping from zero mV holding potential to ± 100 mV. Representative tracings are the average of $n=12$ cells. The tracings on the left are the control, followed by the whole-cell tracings while perfusing the test solution (middle). The tracings on the right are the composite delta obtained by subtraction of the test average values, from those under control conditions. The whole-cell conductance, obtained from the current-to-voltage relationships is highly linear under both conditions, and reflects a modest, albeit significant change in conductance due to the test conditions. The contribution to the whole-cell conductance, i.e., the component inhibited by the drug (inventive electrokinetically generated fluid) is also linear, and the reversal potential is near zero mV. There is a decrease in the whole cell conductance under hyperpolarizing conditions.

Figure 101 shows whole-cell currents under basal conditions, with a protocol stepping from -40 mV holding potential to ± 100 mV. Representative tracings are the average of $n=12$ cells. The tracings on the left are the control, followed by the whole-cell tracings while perfusing the test solution (middle). The tracings on the right are the composite delta obtained by subtraction of the test average values, from those under control conditions. The whole-cell conductance obtained from the current-to-voltage relationships is highly linear under both conditions, and reflects a modest, albeit significant change in conductance due to the test conditions. The contribution to the whole-cell conductance, i.e., the component inhibited by the drug (inventive electrokinetically generated fluid) is also linear, and the reversal potential is near zero mV. Values are comparatively similar to those obtained with the zero mV protocol.

Figure 102 shows whole-cell currents under basal conditions, with a protocol stepping from -60 mV holding potential to ± 100 mV. Representative tracings are the average of $n=12$ cells. The tracings on the left are the control, followed by the whole-cell tracings while perfusing the test solution (middle). The tracings on the right are the composite delta obtained by subtraction of the test average values, from those under control conditions. The whole-cell conductance obtained from the current -to-voltage relationships is highly linear under both conditions, and reflects a minor, albeit significant change in conductance due to the test conditions. The contribution to the whole-cell conductance, i.e., the component inhibited by the drug is also linear, and the

reversal potential is near zero mV. Values are comparatively similar to those obtained with the zero mV protocol.

Figure 103 shows whole-cell currents under basal conditions, with a protocol stepping from -120 mV holding potential to ± 100 mV. Representative tracings are the average of $n=12$ cells. The tracings on the left are the control, followed by the whole-cell tracings while perfusing the test solution (middle). The tracings on the right are the composite delta obtained by subtraction of the test average values, from those under control conditions. The whole-cell conductance obtained from the current -to-voltage relationships is highly linear under both conditions, and reflects a minor, albeit significant change in conductance due to the test conditions. The contribution to the whole-cell conductance, i.e., the component inhibited by the drug is also linear, and the reversal potential is near zero mV. Values are comparatively similar to those obtained with the zero mV protocol.

Figure 104 shows whole-cell currents under cAMP-stimulated conditions, obtained with protocols stepping from various holding potentials to ± 100 mV. Representative tracings are the average of $n=5$ cells. The tracings on the left are the control, followed by the whole-cell tracings after cAMP stimulation, followed by perfusion with the drug-containing solution. The tracings on the right are the composite delta obtained by subtraction of the test average values in drug + cAMP, from those under control conditions (cAMP alone). The tracings on the Top are those obtained from voltage protocol at zero mV, and the ones below, at -40 mV. The whole-cell conductance obtained from the current-to-voltage relationships is highly linear under all conditions, and reflects a change in conductance due to the test conditions.

Figure 105 shows whole-cell currents under cAMP-stimulated conditions, obtained with protocols stepping from various holding potentials to ± 100 mV. Representative tracings are the average of $n=5$ cells. The tracings on the left are the control, followed by the whole-cell tracings after cAMP stimulation, followed by perfusion with the drug-containing solution. The tracings on the right are the composite delta obtained by subtraction of the test average values in drug + cAMP, from those under control conditions (cAMP alone). The tracings on the Top are those obtained from voltage protocol at -60 mV, and the ones below, at -120 mV. The whole-cell conductance, obtained from the current-to-voltage relationships, is highly linear under all conditions, and reflects a change in conductance due to the test conditions.

Figure 106 shows the effect of holding potential on cAMP-activated currents. The effect of the drug (the inventive electrokinetically generated fluids; RNS-60; electrokinetically treated normal saline comprising 60 ppm dissolved oxygen) on the whole-cell conductance was observed under different voltage protocols (0, -40, -60, -120 mV holding potentials). Under basal conditions, the drug-sensitive whole-cell current was identical at all holding potentials (voltage-insensitive contribution, Top Left panel). In the cAMP-activated conditions, however, the drug-sensitive currents were much higher, and sensitive to the applied voltage protocol. The current-to-voltage relationships are highly nonlinear. This is further observed in the subtracted currents (Bottom panel), where the contribution of the whole cell conductance at zero mV was further subtracted for each protocol (n=5).

Summary of Example. According to particular aspects, therefore, the data indicate that there is a modest but consistent effect of the drug (the inventive electrokinetically generated fluids; RNS-60; electrokinetically treated normal saline comprising 60 ppm dissolved oxygen) under basal conditions. To enhance the effect of the drug on the whole-cell conductance, experiments were also conducted by perfusing the drug after stimulation with a cAMP stimulating “cocktail”, which dramatically increased the whole-cell conductance. Interestingly, this protocol also increased the drug-sensitive portion of the whole-cell conductance, which was *ten-times* higher than that observed under basal conditions. Additionally, in the presence of cAMP stimulation, the drug showed different effects with respect to the various voltage protocols, indicating that *the electrokinetically generated fluids affect a voltage-dependent contribution of the whole-cell conductance*. There was also a decrease in a linear component of the conductance, further suggesting at least a contribution of the drug to the inhibition of another pathway (e.g., ion channel, voltage gated cation channels, etc.).

In particular aspects, and without being bound by mechanism, Applicants' data are consistent with the inventive electrokinetically generated fluids (e.g., RNS-60; electrokinetically treated normal saline comprising 60 ppm dissolved oxygen) producing a change either on a channel(s), being blocked or retrieved from the plasma membrane.

Taken together with Applicants' other data (e.g., the data of working Examples) particular aspects of the present invention provide compositions and methods for

modulating intracellular signal transduction, including modulation of at least one of membrane structure, membrane potential or membrane conductivity, membrane proteins or receptors, ion channels, and calcium dependant cellular signalling systems, comprising use of the inventive electrokinetically generated solutions to impart electrochemical and/or conformational changes in membranous structures (e.g., membrane and/or membrane proteins, receptors or other components) including but not limited to GPCRs and/or g-proteins. According to additional aspects, these effects modulate gene expression, and may persist, dependant, for example, on the half lives of the individual messaging components, etc.

Incorporation by Reference

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety.

It should be understood that the drawings and detailed description herein are to be regarded in an illustrative rather than a restrictive manner, and are not intended to limit the invention to the particular forms and examples disclosed. On the contrary, the invention includes any further modifications, changes, rearrangements, substitutions, alternatives, design choices, and embodiments apparent to those of ordinary skill in the art, without departing from the spirit and scope of this invention, as defined by the following claims. Thus, it is intended that the following claims be interpreted to embrace all such further modifications, changes, rearrangements, substitutions, alternatives, design choices, and embodiments.

The foregoing described embodiments depict different components contained within, or connected with, different other components. It is to be understood that such depicted architectures are merely exemplary, and that in fact many other architectures can be implemented which achieve the same functionality. In a conceptual sense, any arrangement of components to achieve the same functionality is effectively "associated" such that the desired functionality is achieved. Hence, any two components herein combined to achieve a particular functionality can be seen as "associated with" each other such that the desired functionality is achieved, irrespective of architectures or intermedial components. Likewise, any two components so associated can also be

viewed as being "operably connected", or "operably coupled", to each other to achieve the desired functionality.

While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that, based upon the teachings herein, changes and modifications may be made without departing from this invention and its broader aspects and, therefore, the appended claims are to encompass within their scope all such changes and modifications as are within the true spirit and scope of this invention. Furthermore, it is to be understood that the invention is solely defined by the appended claims. It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (e.g., bodies of the appended claims) are generally intended as "open" terms (e.g., the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases "at least one" and "one or more" to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles "a" or "an" limits any particular claim containing such introduced claim recitation to inventions containing only one such recitation, even when the same claim includes the introductory phrases "one or more" or "at least one" and indefinite articles such as "a" or "an" (e.g., "a" and/or "an" should typically be interpreted to mean "at least one" or "one or more"); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation *is* explicitly recited, those skilled in the art will recognize that such recitation should typically be interpreted to mean *at least* the recited number (e.g., the bare recitation of "two recitations," without other modifiers, typically means *at least* two recitations, or *two or more* recitations). Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

1. A method for modulating intracellular signal transduction, comprising contacting at least one cell having a membrane and membrane components with an electrokinetically altered aqueous fluid, the electrokinetically altered aqueous fluid suitable to alter cellular membrane structure or function sufficient to provide for modulation of intracellular signal transduction.

2. The method of claim 1, wherein alteration of the electrokinetically altered aqueous fluid comprises exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects.

3. The method of claim 2, wherein, exposure to the localized electrokinetic effects comprises exposure to at least one of voltage pulses and current pulses.

4. The method of claim 2, wherein the exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects, comprises exposure of the fluid to electrokinetic effect-inducing structural features of a device used to generate the fluid.

5. The method of claim 1, wherein altering cellular membrane structure or function comprises altering of a conformation, ligand binding activity, or a catalytic activity of a membrane associated protein.

6. The method of claim 5, wherein the membrane associated protein comprises at least one selected from the group consisting of receptors, transmembrane receptors, ion channel proteins, intracellular attachment proteins, cellular adhesion proteins, integrins, etc.

7. The method of claim 6, wherein the transmembrane receptor comprises a G-Protein Coupled Receptor (GPCR).

8. The method of claim 7, wherein the G-Protein Coupled Receptor (GPCR) interacts with a G protein a subunit.

9. The method of claim 8, wherein the G protein a subunit comprises at least one selected from the group consisting of G_{α_s} , G_{α_i} , G_{α_q} , and $G_{\alpha_{12}}$.

10. The method of claim 9, wherein the at least one G protein a subunit is G_{α_q} .

11. The method of claim 1, wherein altering cellular membrane structure or function comprises altering membrane potential or membrane conductivity.

12. The method of claim 1, wherein modulation of intracellular signal transduction comprises modulation of a calcium dependant cellular messaging pathway or system.

13. The method of claim 1, wherein modulation of intracellular signal transduction comprises modulation of phospholipase C activity.

14. The method of claim 1, wherein modulation of intracellular signal transduction comprises modulation of adenylate cyclase (AC) activity.

15. The method of claim 1, wherein modulation of intracellular signal transduction comprises modulation of intracellular signal transduction associated with at least one condition or symptom selected from the group consisting of inflammation, asthma, neurodegeneration, abnormalities of the brain, central nervous system disruption or degradation, Alzheimer's Disease, aging, developmental abnormalities of bone, altered bone growth, hormone resistance, pseudohypoparathyroidism, hormone hypersecretion, McCune-Albright syndrome, retinal disorders, endocrine disorders, metabolic disorders, developmental disorders, alterations in pigmentation of the skin, premature sexual development, psychological maladies, lung constriction, bronchial constriction, alveolar constriction, metabolic symptoms, insulin resistance, and retinal disruption or degradation.

16. The method of claim 1, comprising a cell network or layer, and further comprising modulation of an intercellular junction therein.

17. The method of claim 16, wherein the intracellular junction comprises at least one selected from the group consisting of tight junctions, gap junctions, zona adherins and desmosomes.

18. The method of claim 1, wherein the cell network or layers comprises at least one selected from the group consisting of pulmonary epithelium, bronchial epithelium, intestinal epithelium, and corneal epithelium.

19. The method of claim 1, wherein the electrokinetically altered aqueous fluid is oxygenated, and wherein the oxygen in the fluid is present in an amount of at least 15, ppm, at least 25 ppm, at least 30 ppm, at least 40 ppm, at least 50 ppm, or at least 60 ppm oxygen at atmospheric pressure.

20. The method of any one of claims 1 through 19, wherein the electrokinetically altered aqueous fluid comprises at least one of solvated electrons, and electrokinetically modified or charged oxygen species.

21. The method of claim 20, wherein the solvated electrons or electrokinetically modified or charged oxygen species are present in an amount of at least 0.01 ppm, at least 0.1 ppm, at least 0.5 ppm, at least 1 ppm, at least 3 ppm, at least 5 ppm, at least 7 ppm, at least 10 ppm, at least 15 ppm, or at least 20 ppm.

22. The method of claim 20, wherein the electrokinetically altered oxygenated aqueous fluid comprises solvated electrons stabilized by molecular oxygen.

23. The method of claim 1, wherein the ability to alter cellular membrane structure or function sufficient to provide for modulation of intracellular signal transduction last for at least two, at least three, at least four, at least five, at least 6 months in a closed container (e.g., closed gas-tight container).

24. A method for modulating cellular membrane conductivity, comprising contacting at least one cell having a membrane and membrane components with an electrokinetically altered aqueous fluid, the electrokinetically altered aqueous fluid suitable to alter cellular membrane structure or function sufficient to provide for modulation of cellular membrane conductivity.

25. The method of claim 24, wherein modulating cellular membrane conductivity, comprises modulating whole-cell conductance.

26. The method of claim 25, wherein modulating whole-cell conductance, comprises modulating at least one voltage-dependent contribution of the whole-cell conductance.

27. The method of claim 24, wherein alteration of the electrokinetically altered aqueous fluid comprises exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects.

28. The method of claim 27, wherein, exposure to the localized electrokinetic effects comprises exposure to at least one of voltage pulses and current pulses.

29. The method of claim 27, wherein the exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects comprises exposure of the fluid to electrokinetic effect-inducing structural features of a device used to generate the fluid.

30. The method of claim 24, wherein modulating cellular membrane conductivity comprises modulation of at least one selected from the group consisting of membrane receptors, transmembrane receptors, ion channel proteins, intracellular attachment proteins, cellular adhesion proteins and integrins.

31. The method of claim 30, wherein modulating cellular membrane conductivity, comprises modulation of ion channel proteins.

32. The method of claim 1, wherein the cell is a mammalian cell.

33. The method of claim 32, wherein the cell is a human cell.

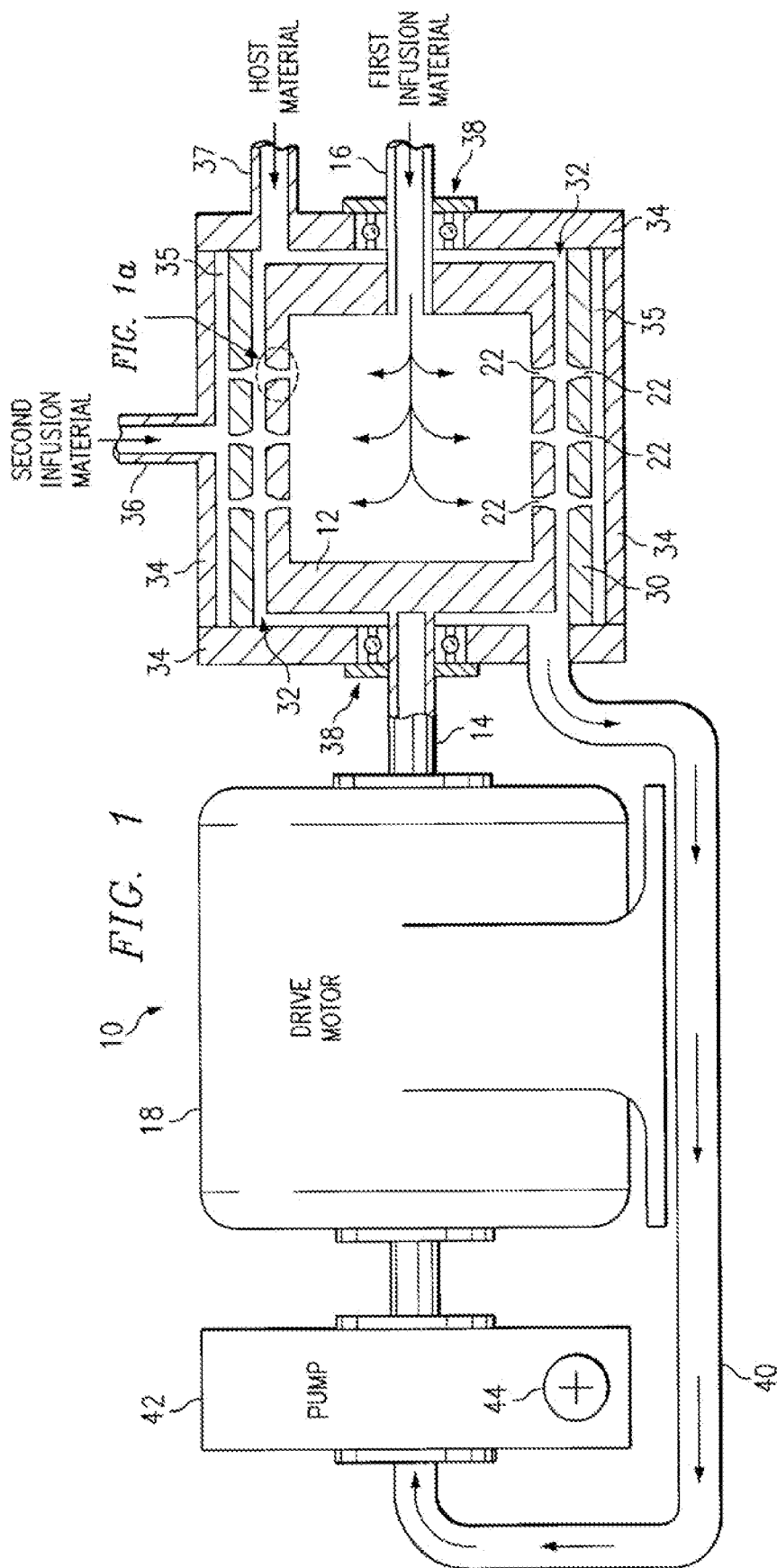


Fig. 1
(Prior Art)

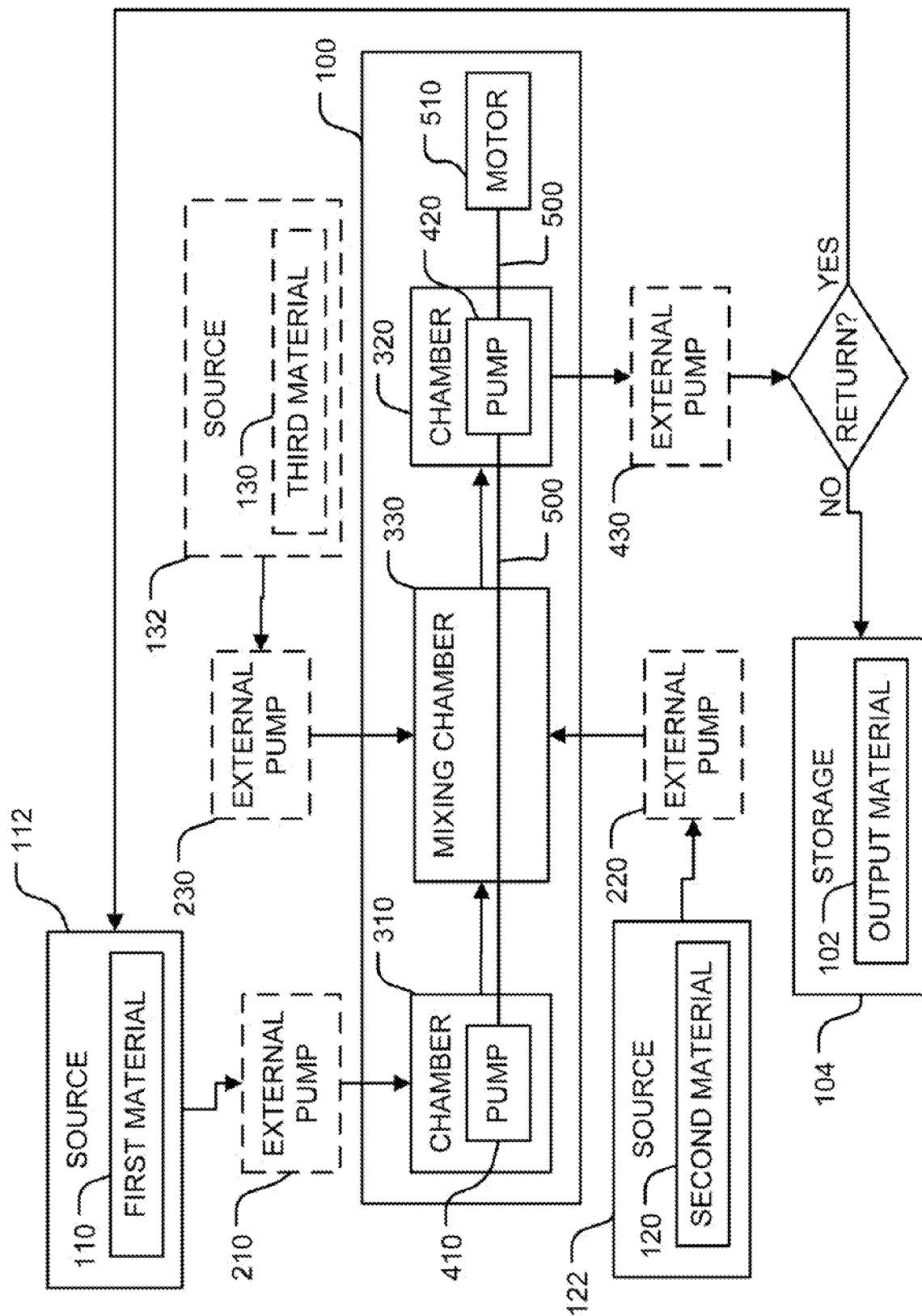


Fig. 2

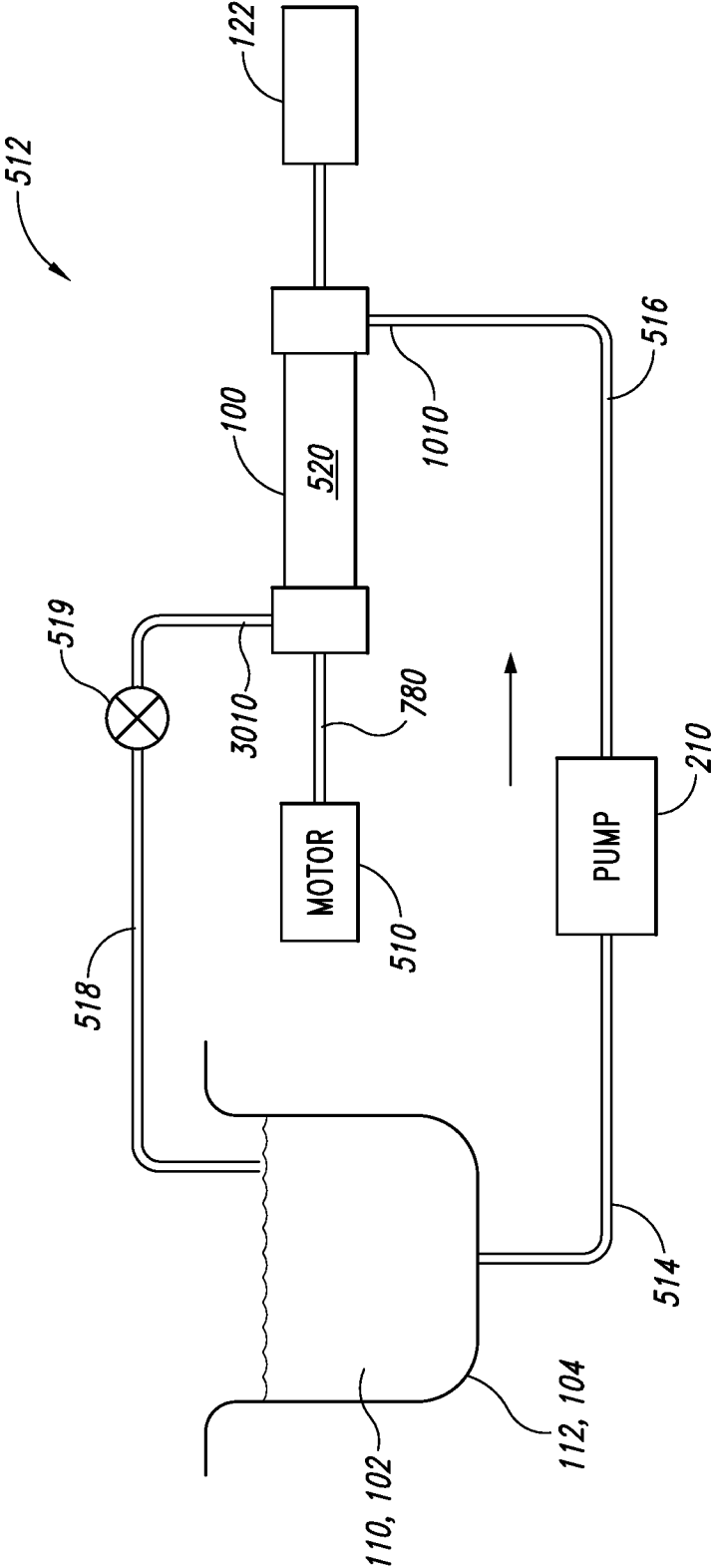


Fig. 3

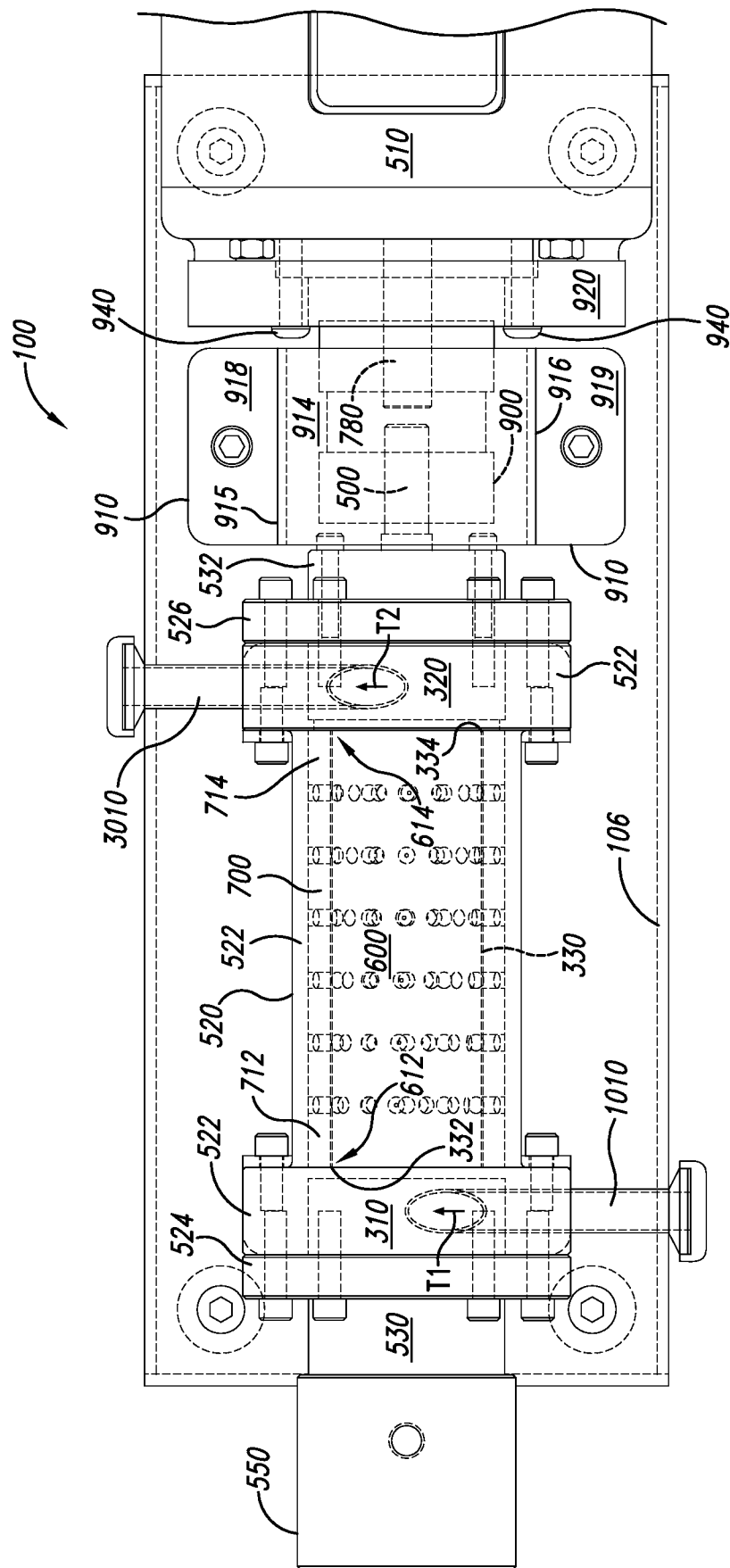


Fig. 4

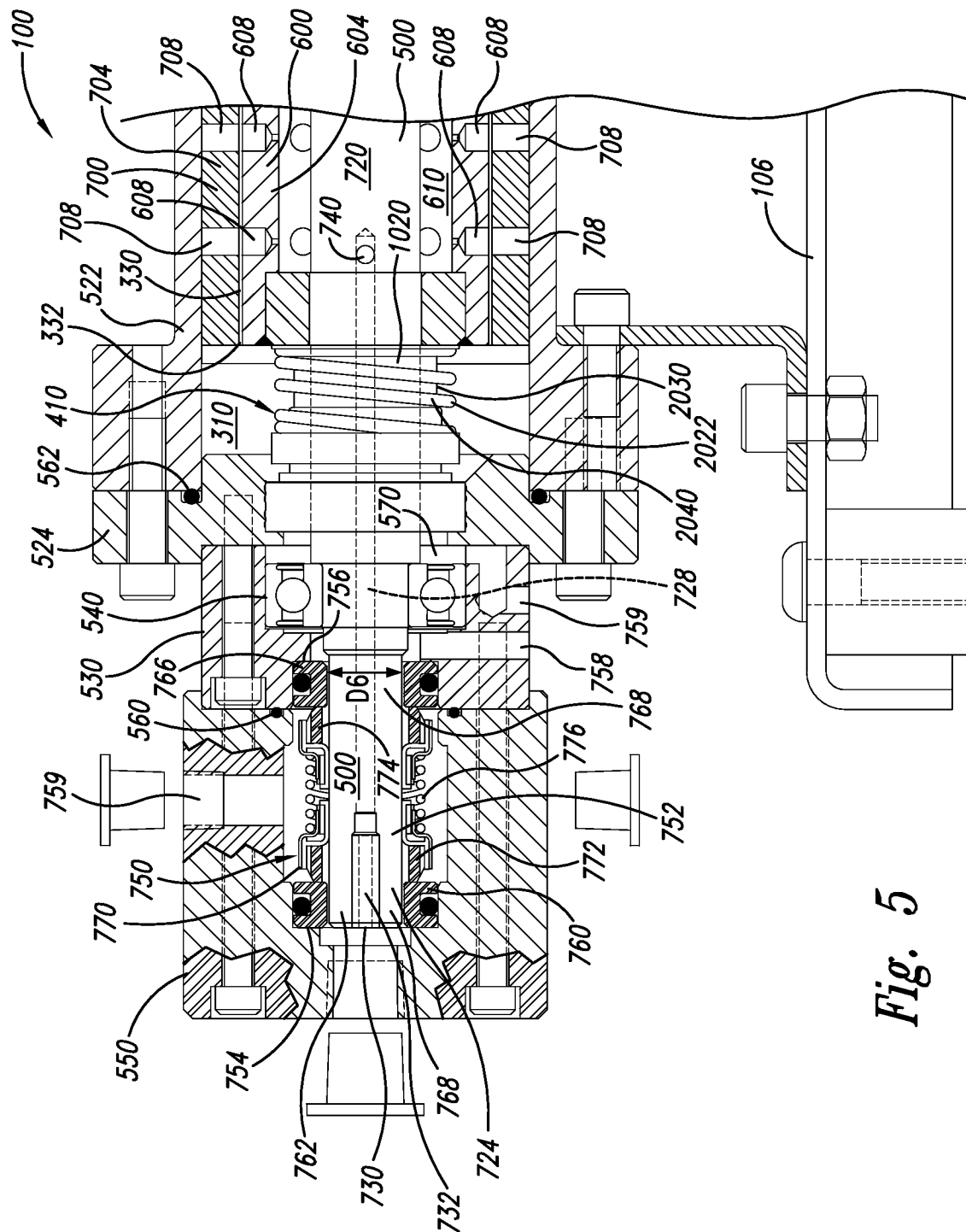


Fig. 5

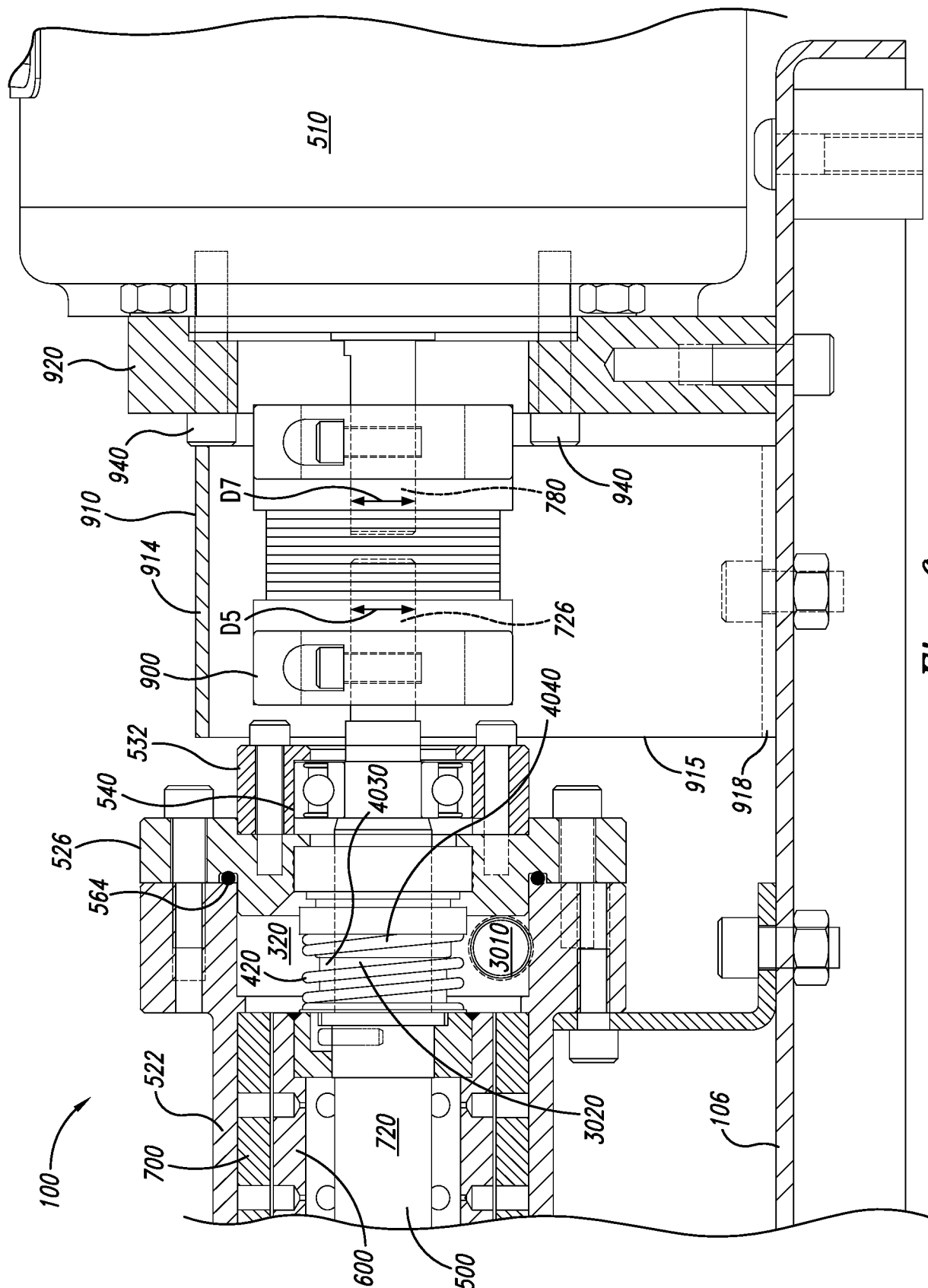


Fig. 6

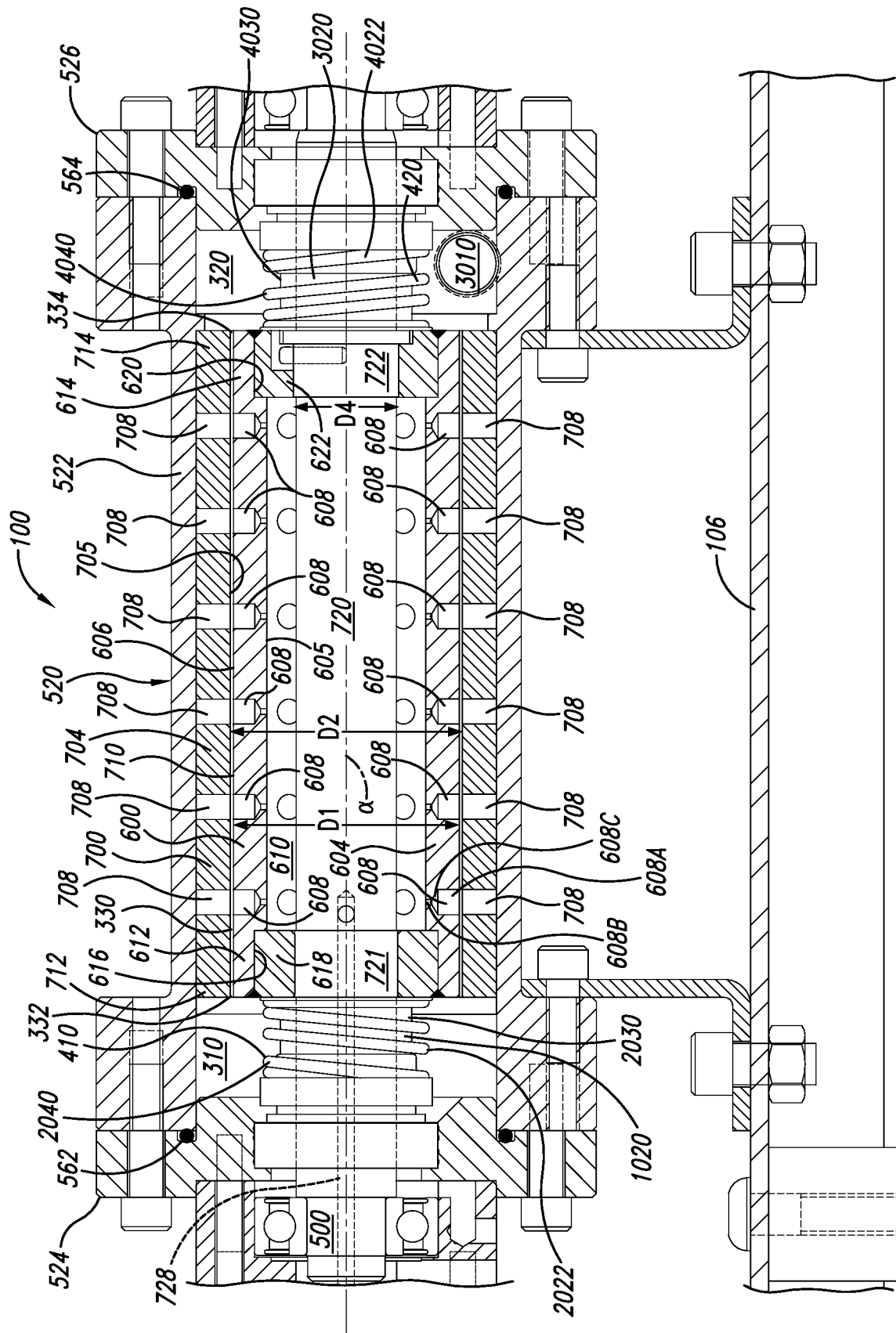


Fig. 7

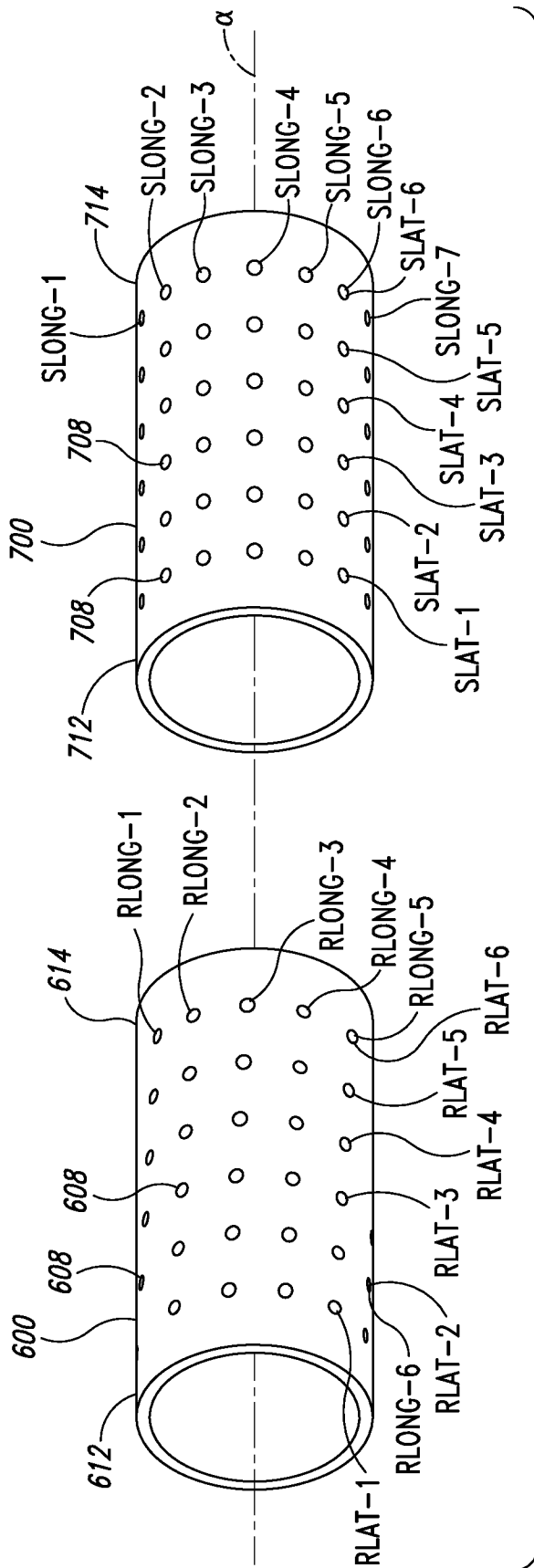


Fig. 8

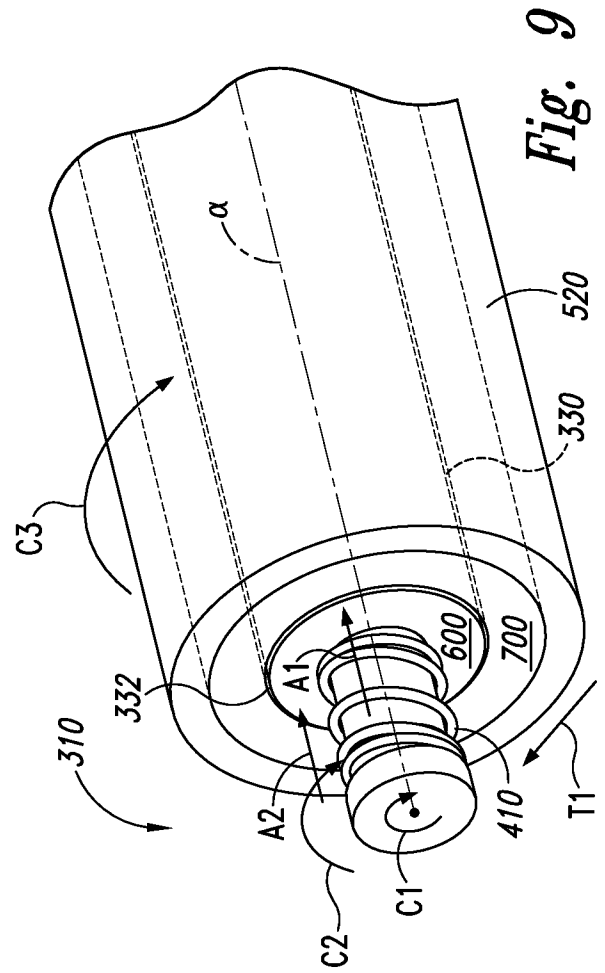


Fig. 9

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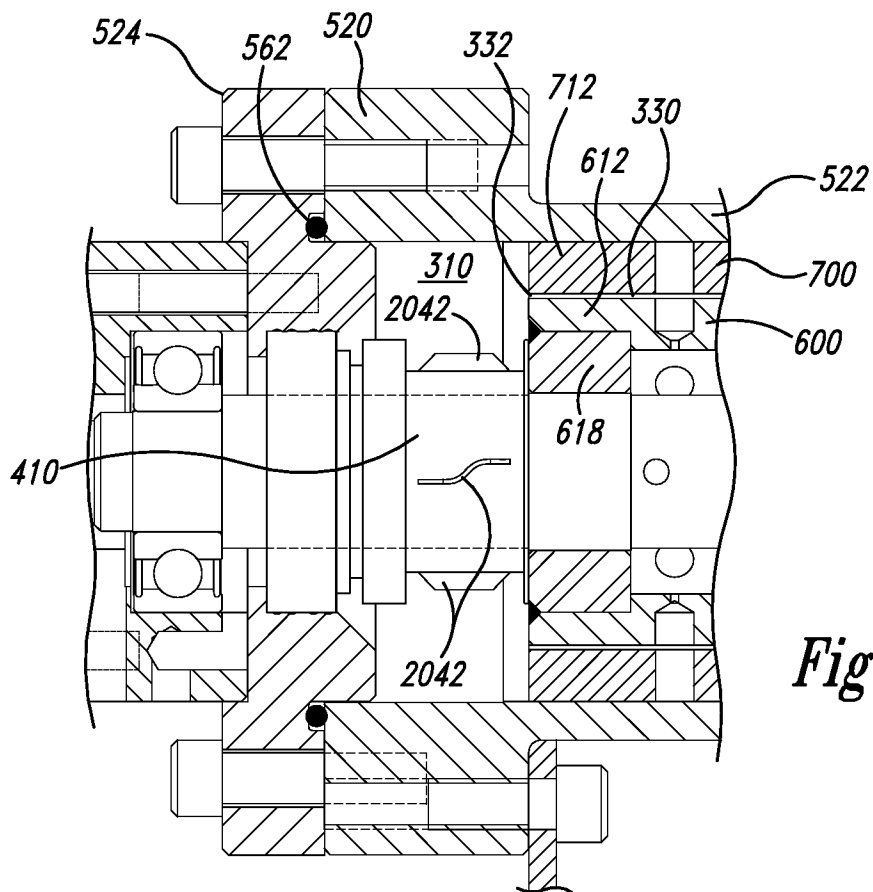


Fig. 10

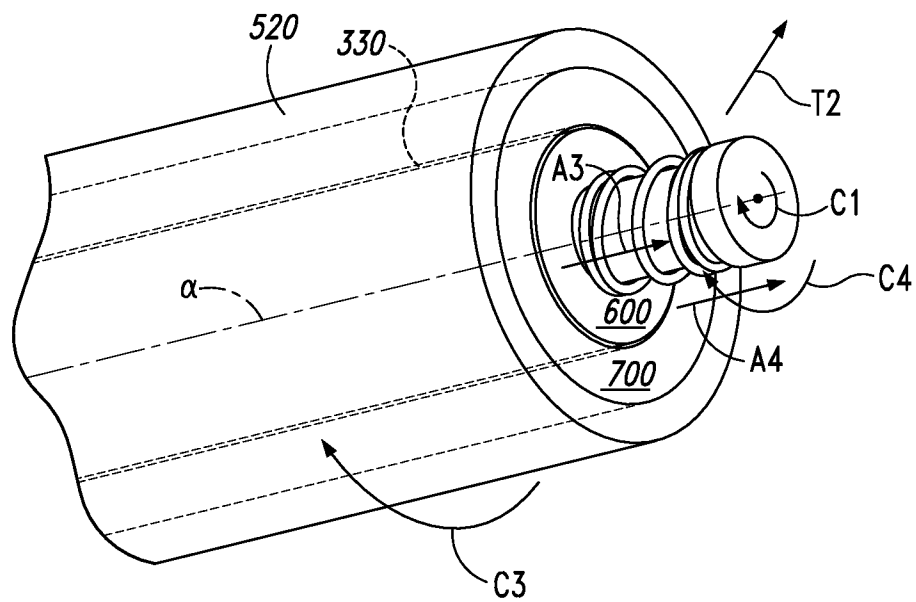


Fig. 11

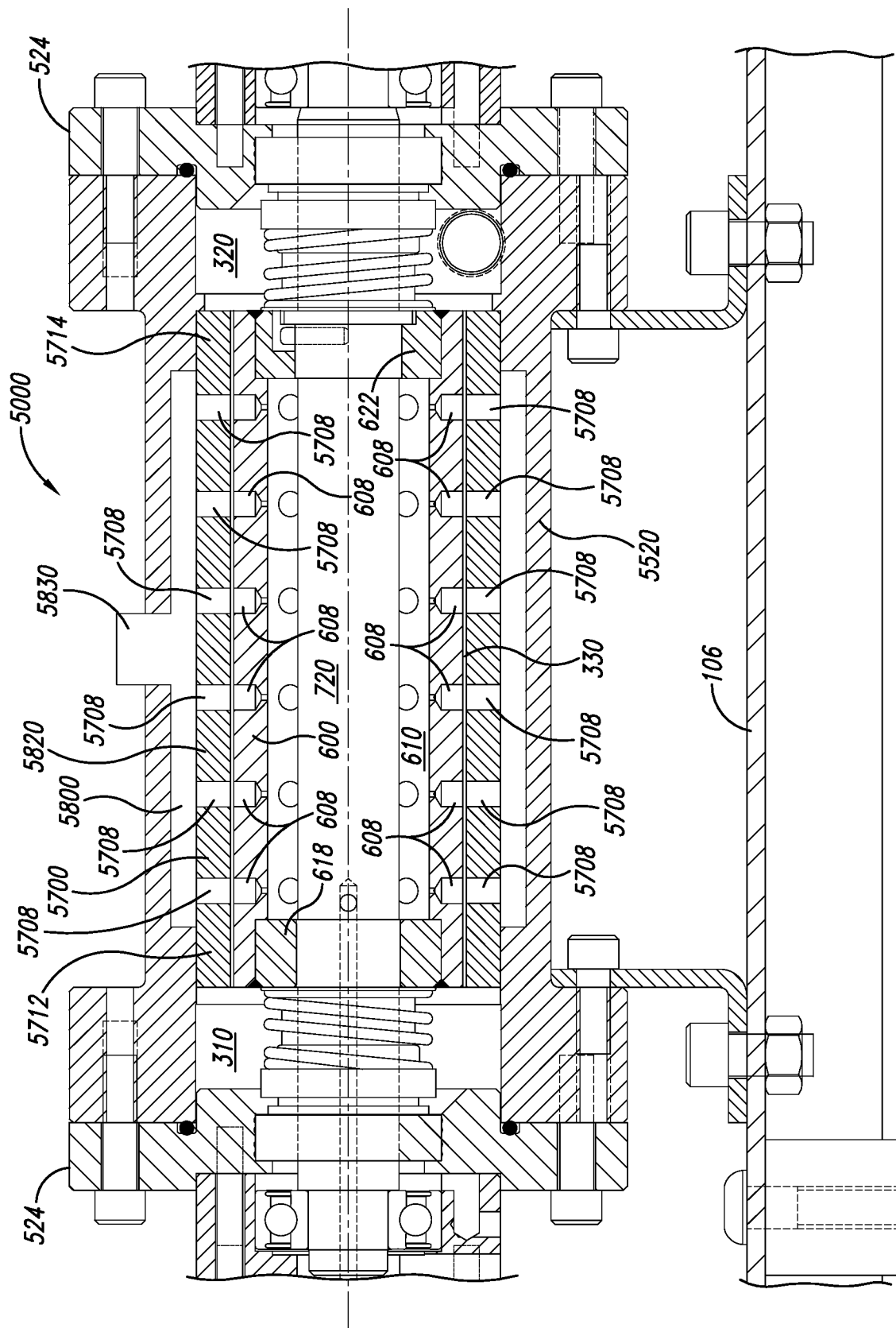
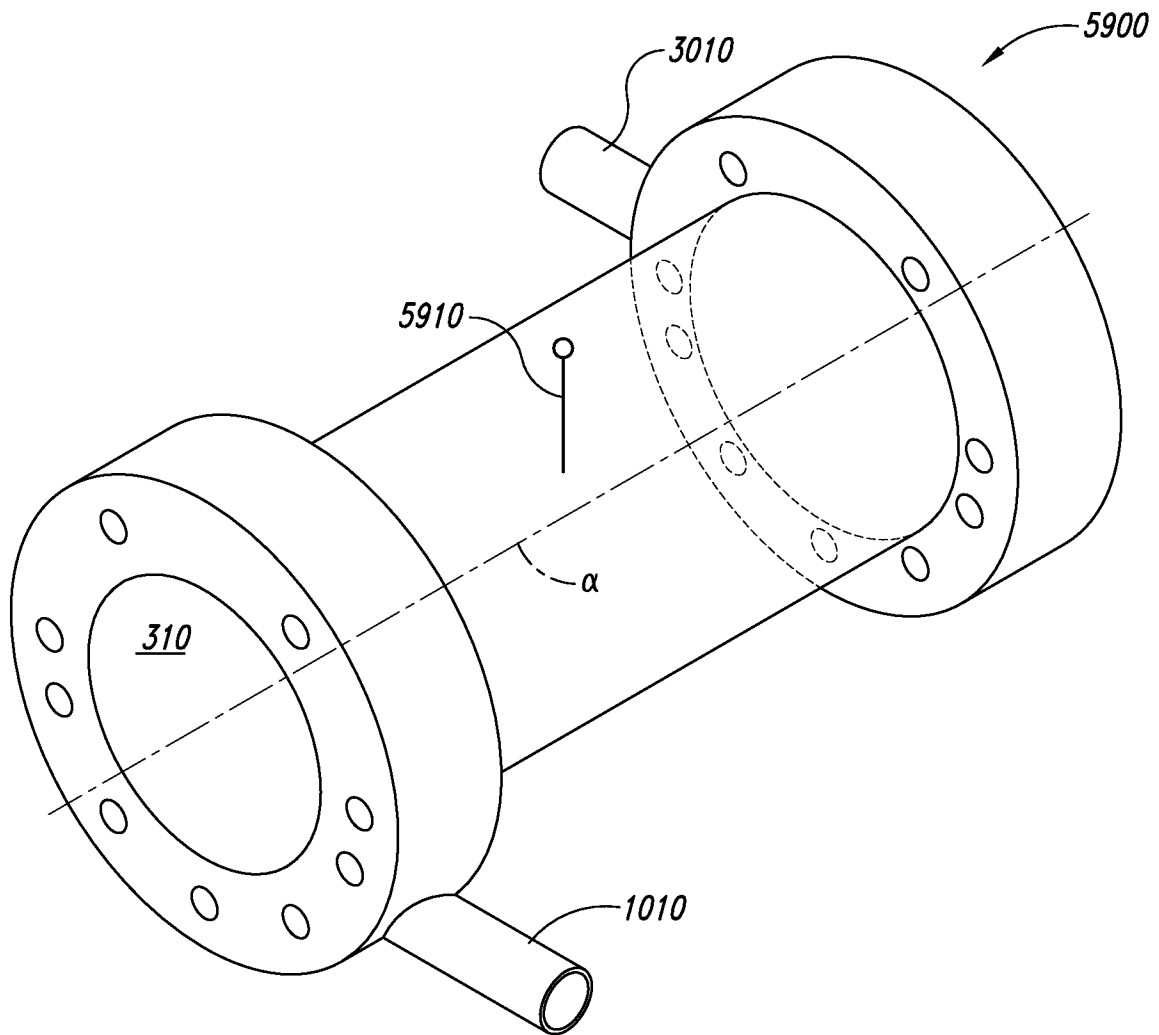


Fig. 12

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*Fig. 13*

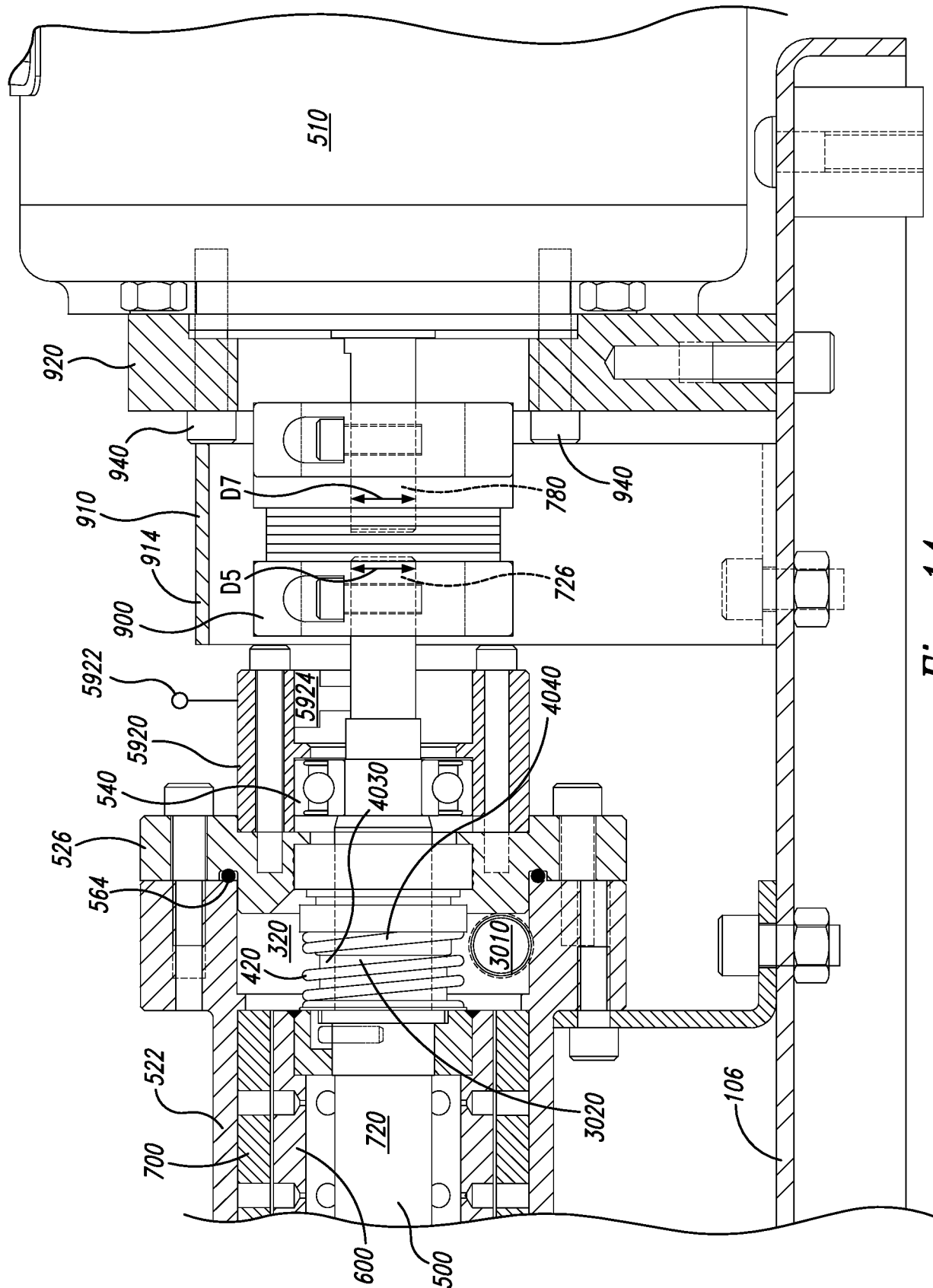


Fig. 14

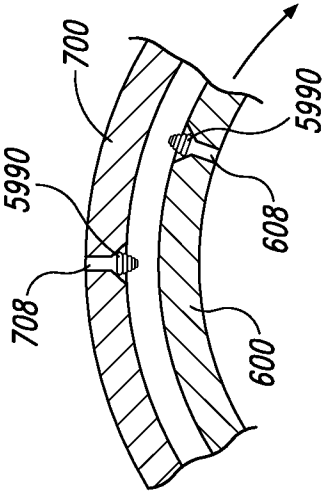


Fig. 15

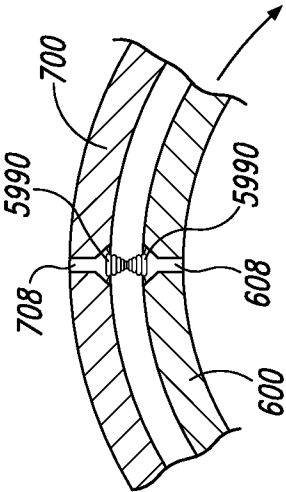


Fig. 16

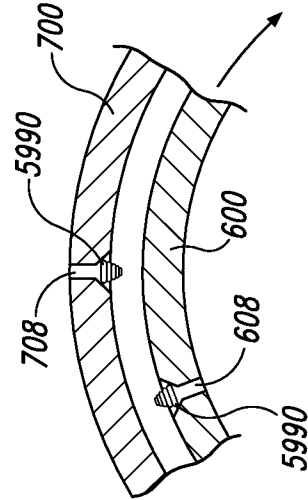


Fig. 17

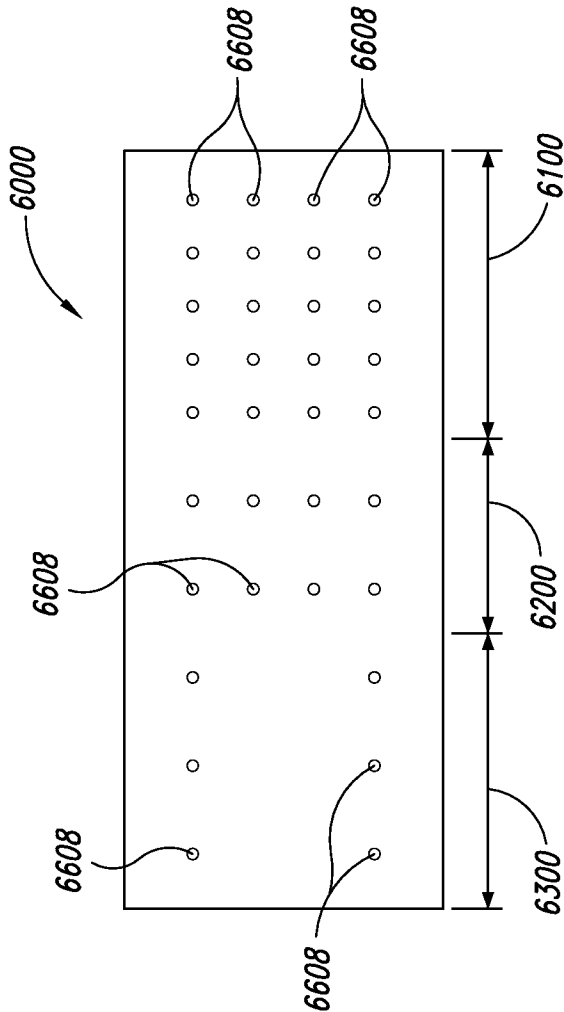


Fig. 18

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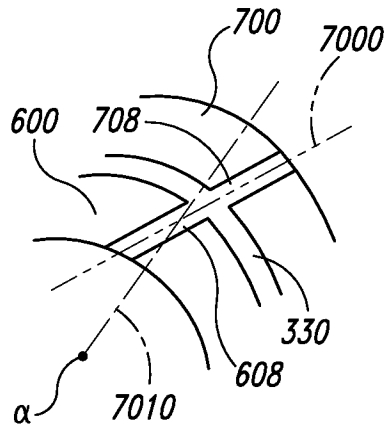


Fig. 19

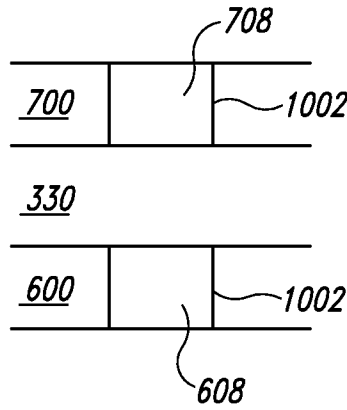


Fig. 20

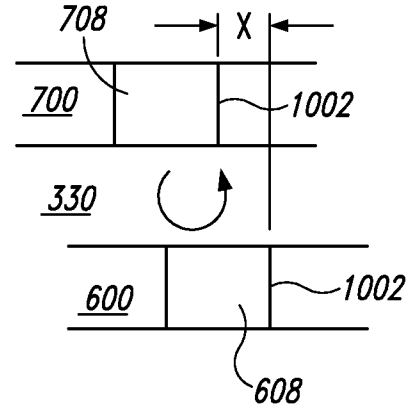


Fig. 21

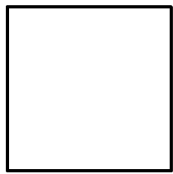


Fig. 22

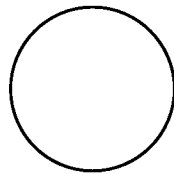


Fig. 23

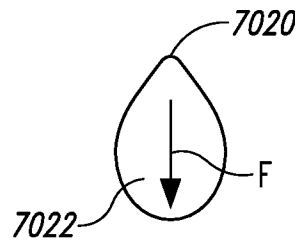


Fig. 24

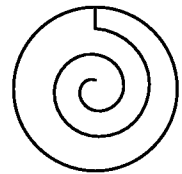


Fig. 25

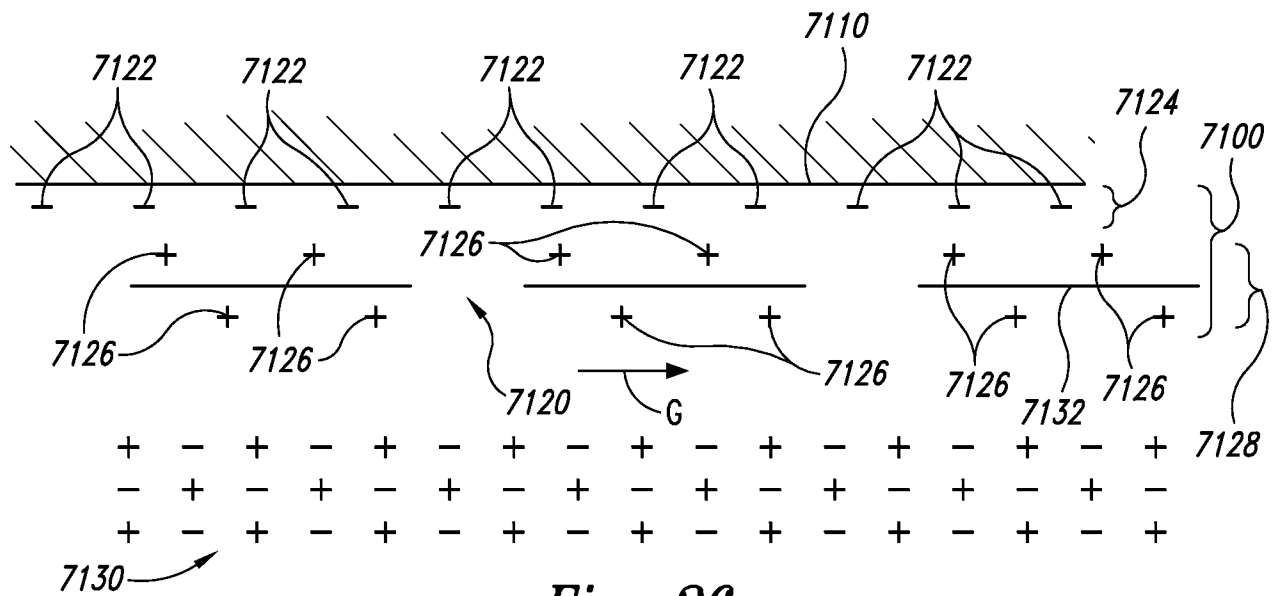
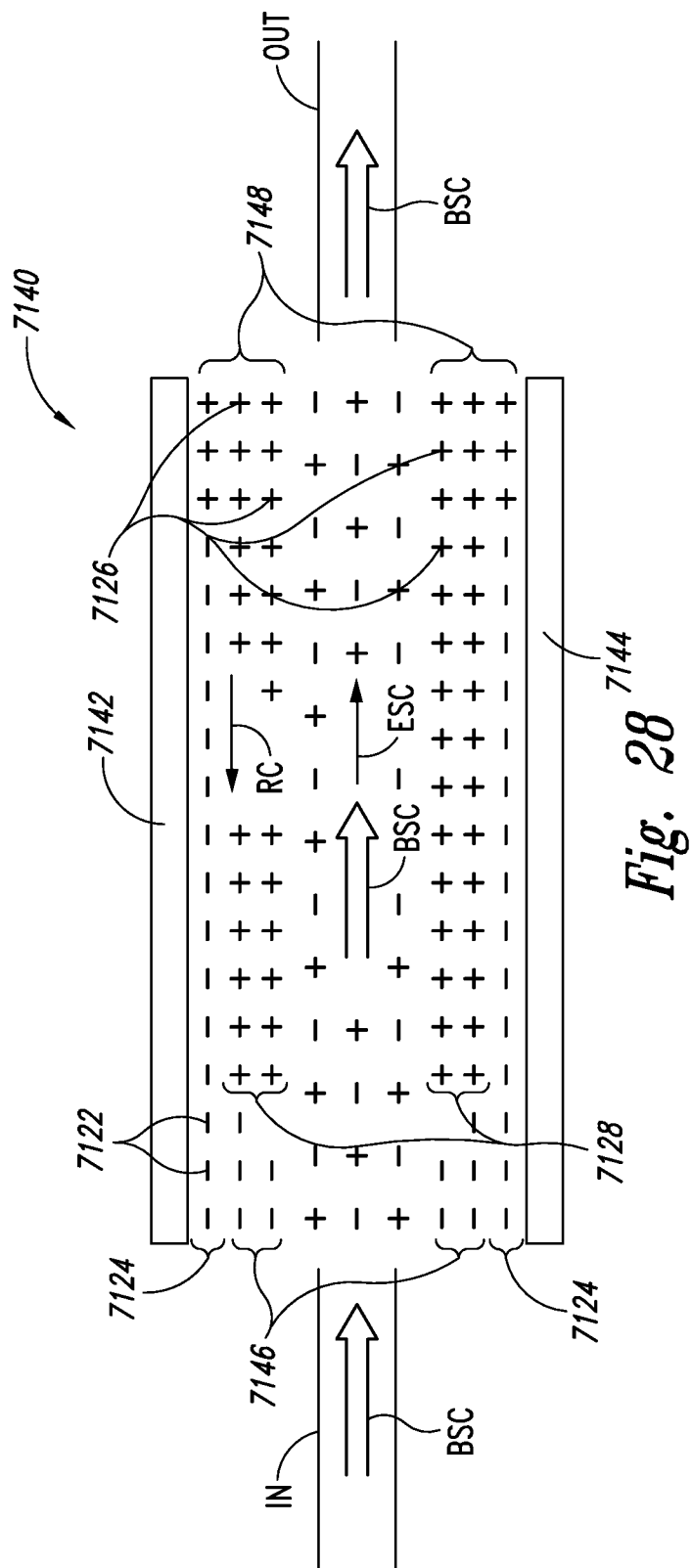
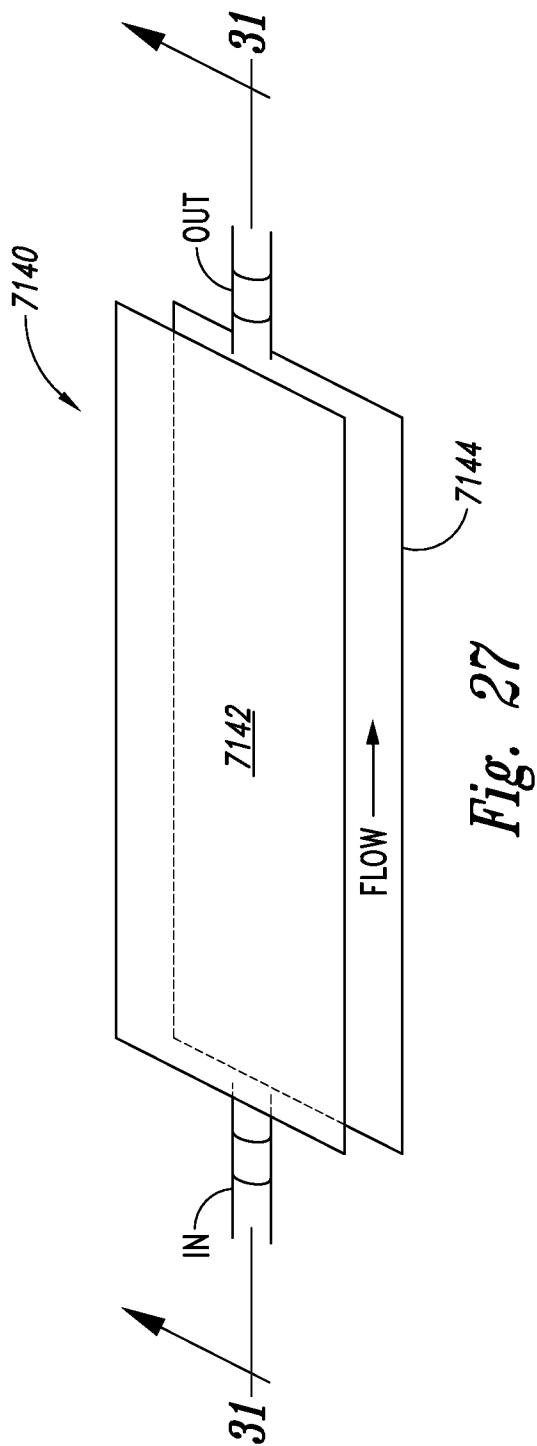
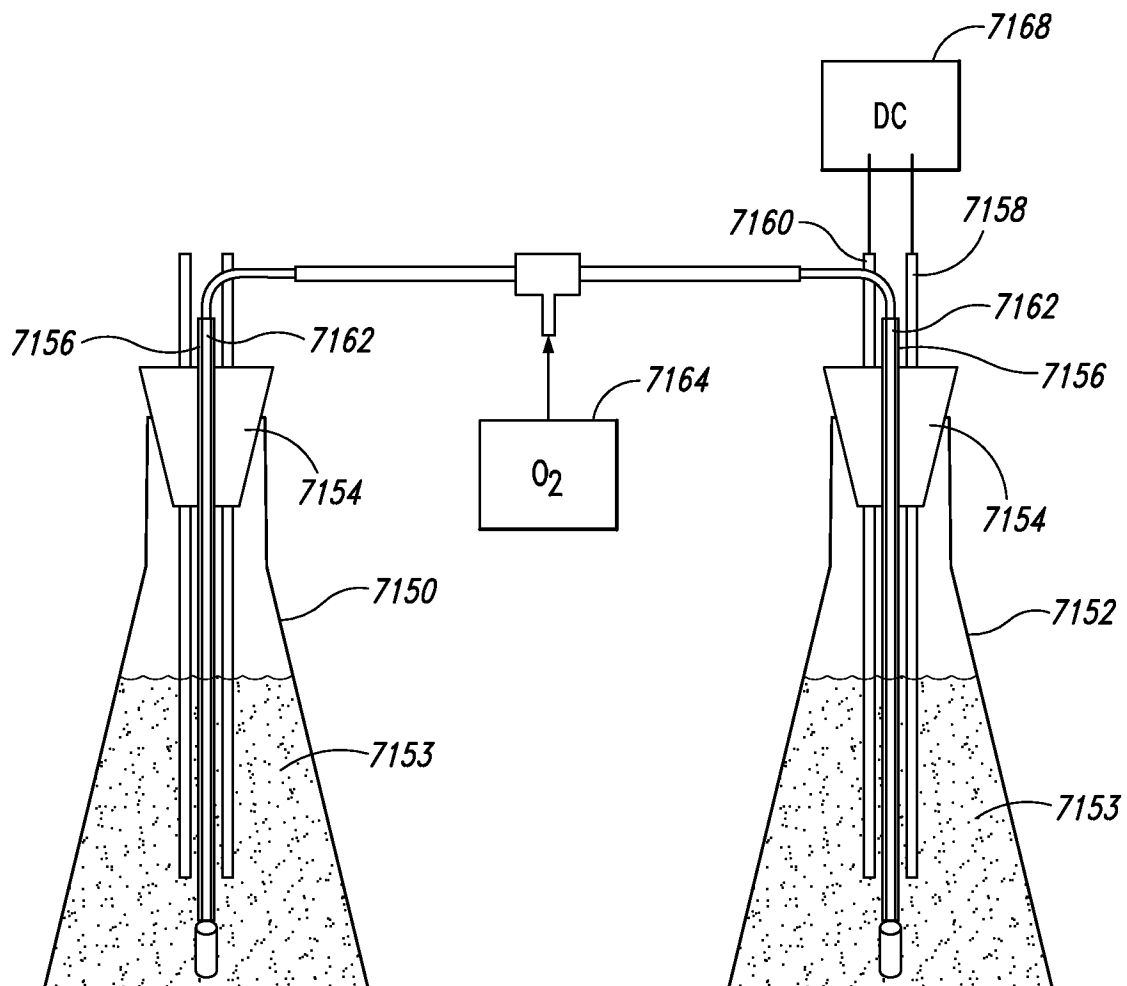
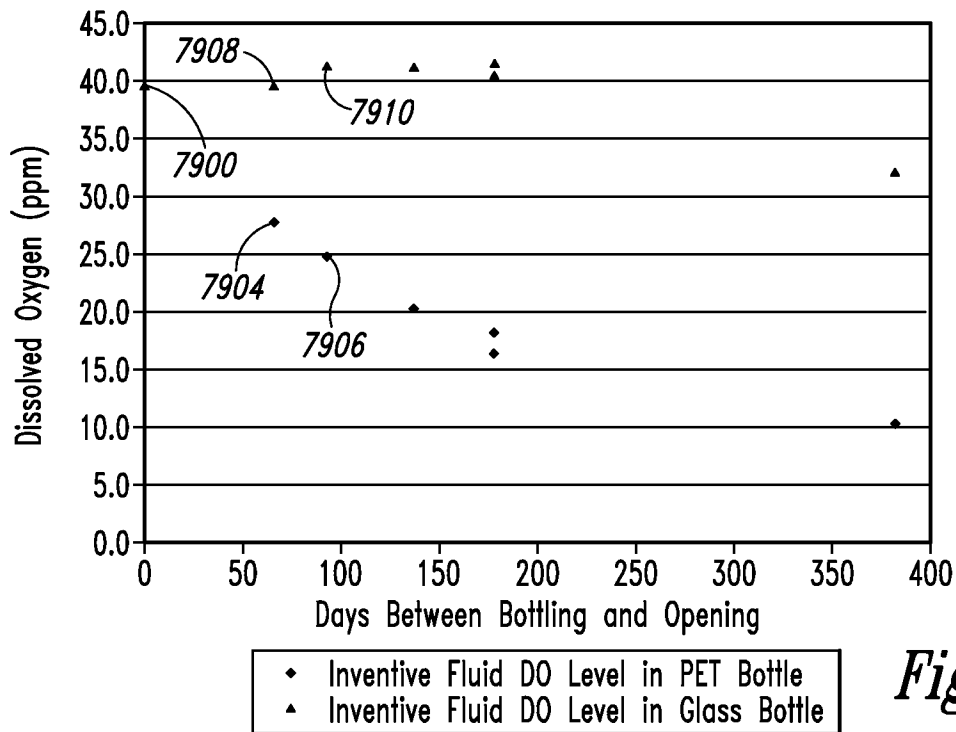
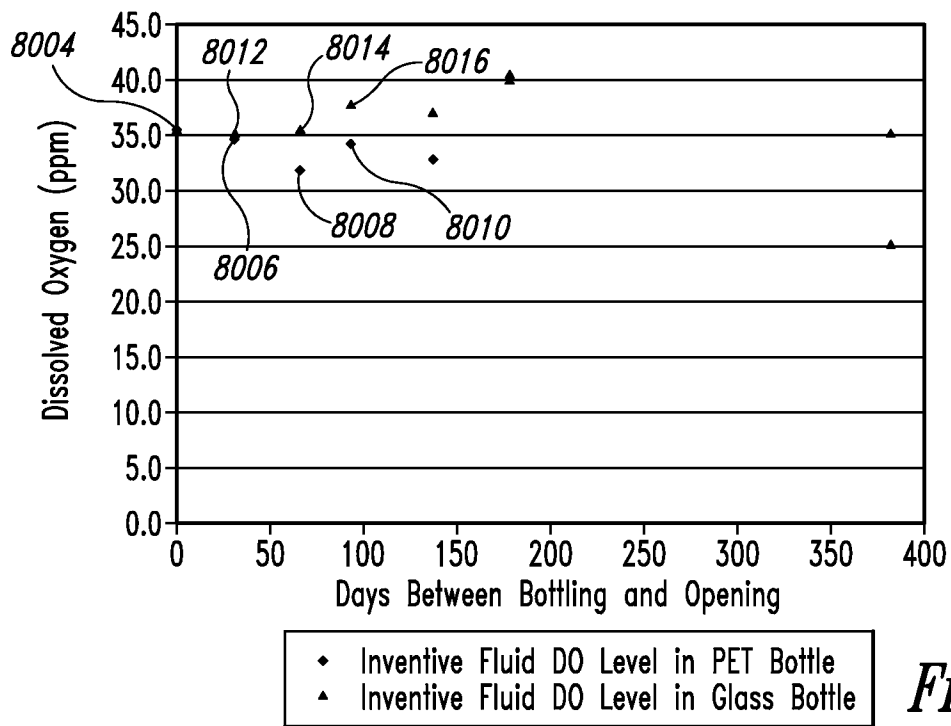


Fig. 26

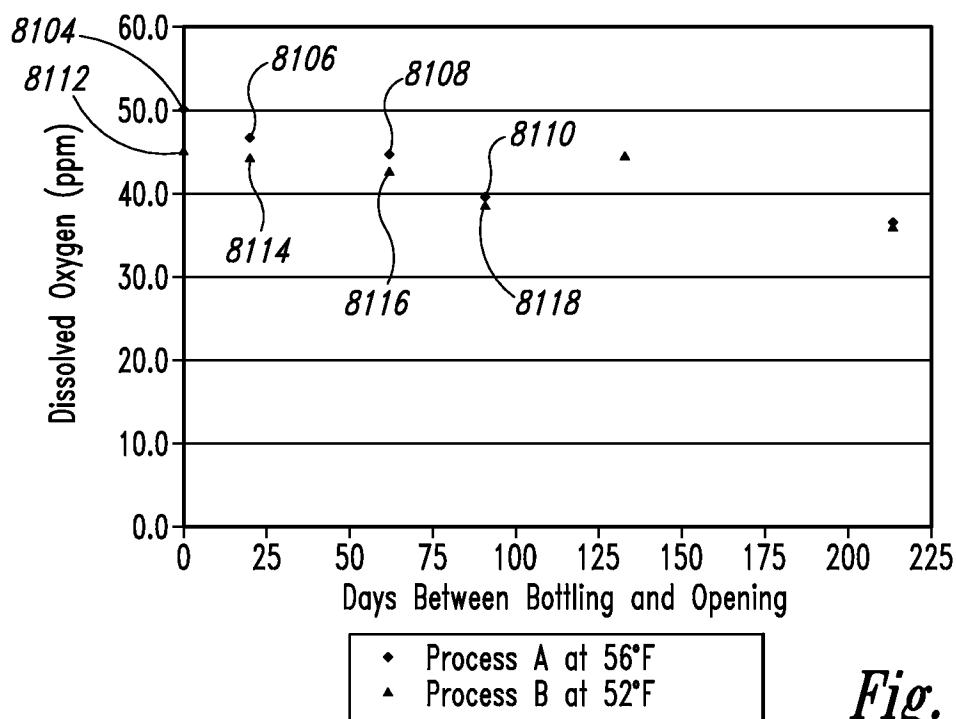
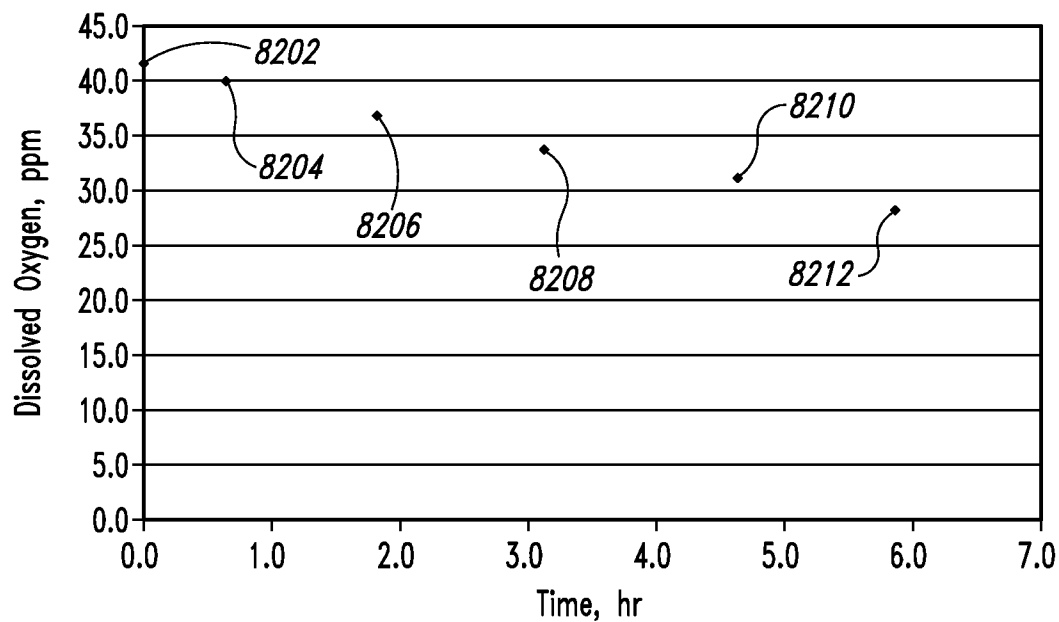


*Fig. 29*

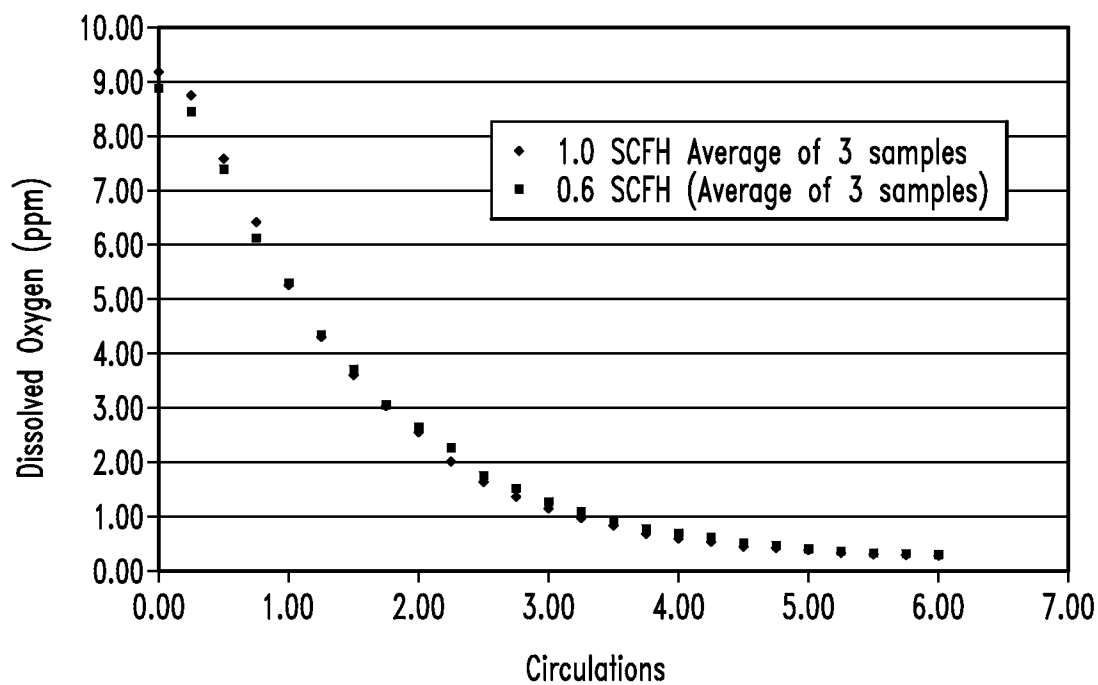
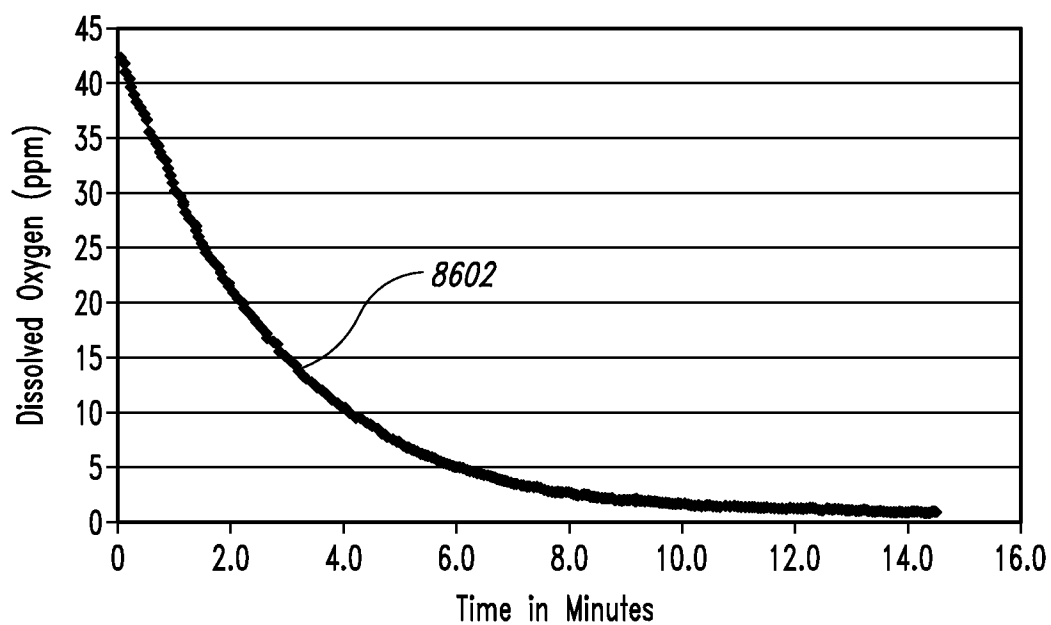
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*Fig. 30**Fig. 31*

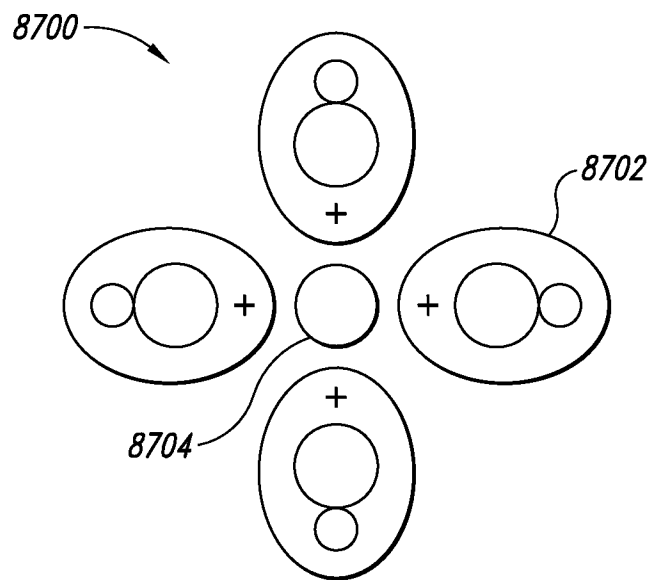
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*Fig. 32**Fig. 33*

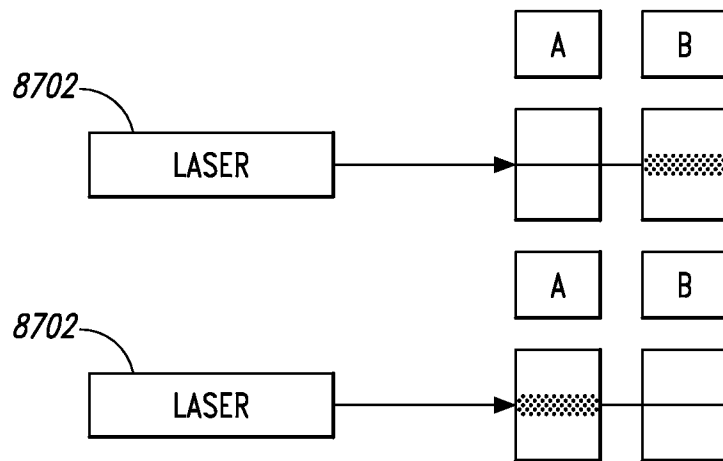
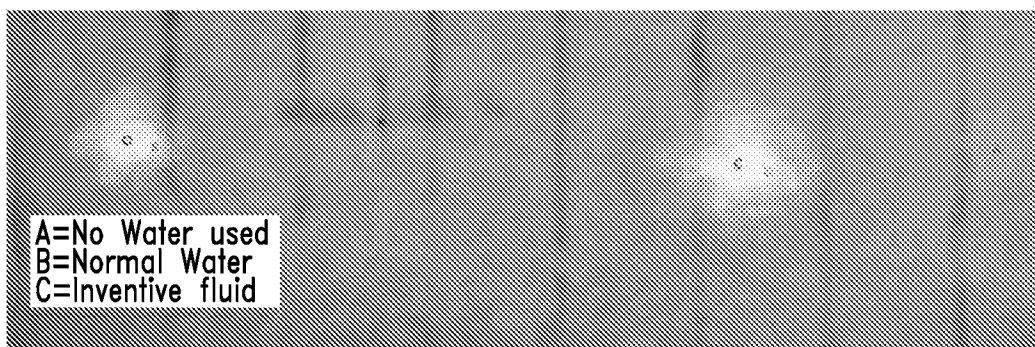
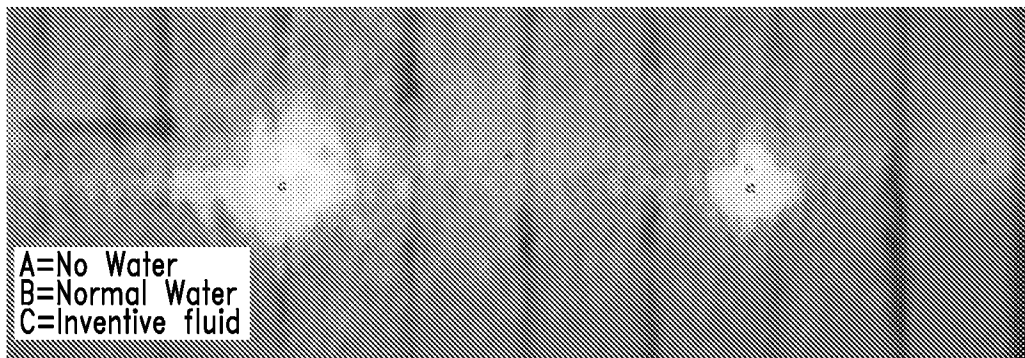
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*Fig. 34**Fig. 35*

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*Fig. 36*

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*Fig. 37A**Fig. 37B*

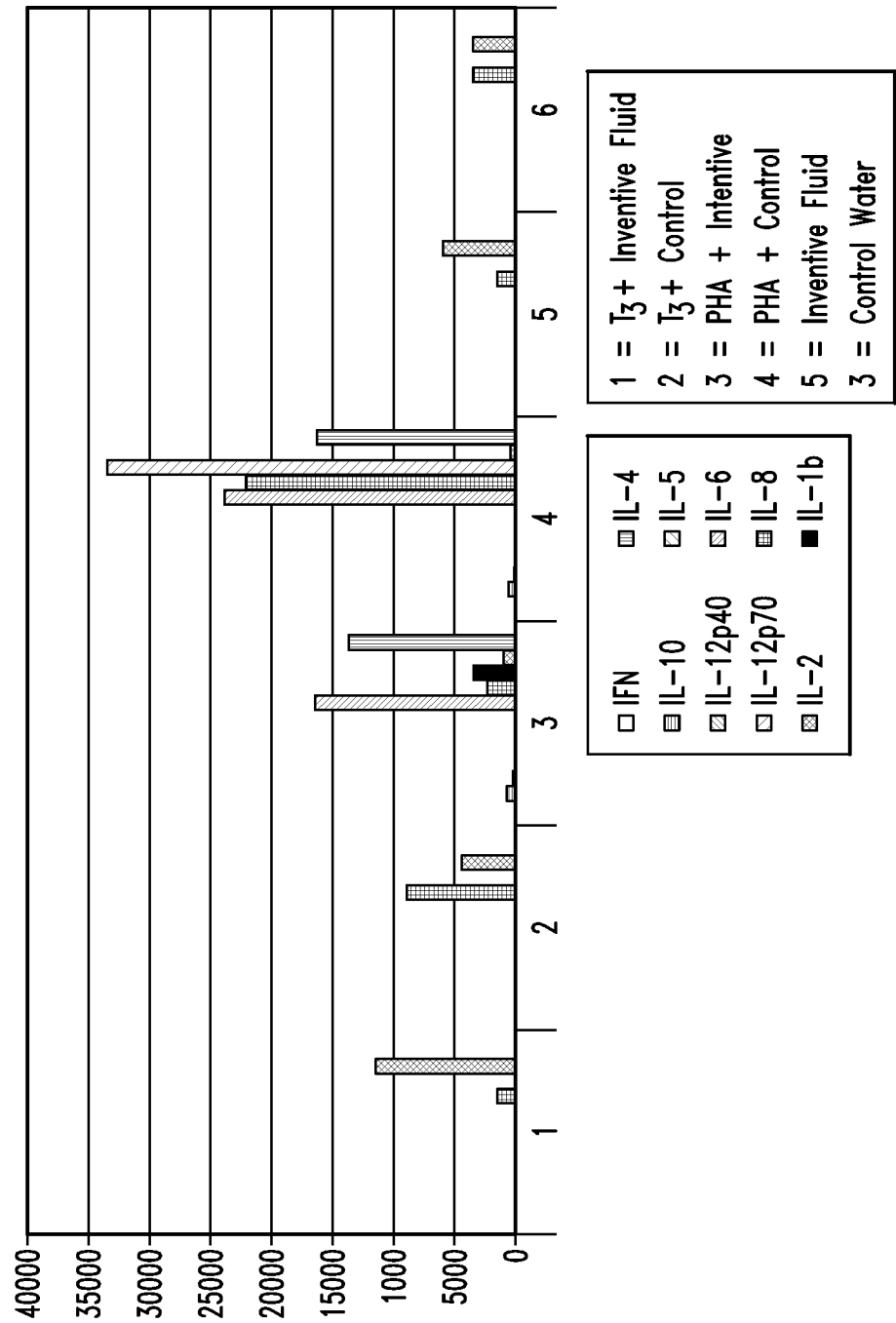
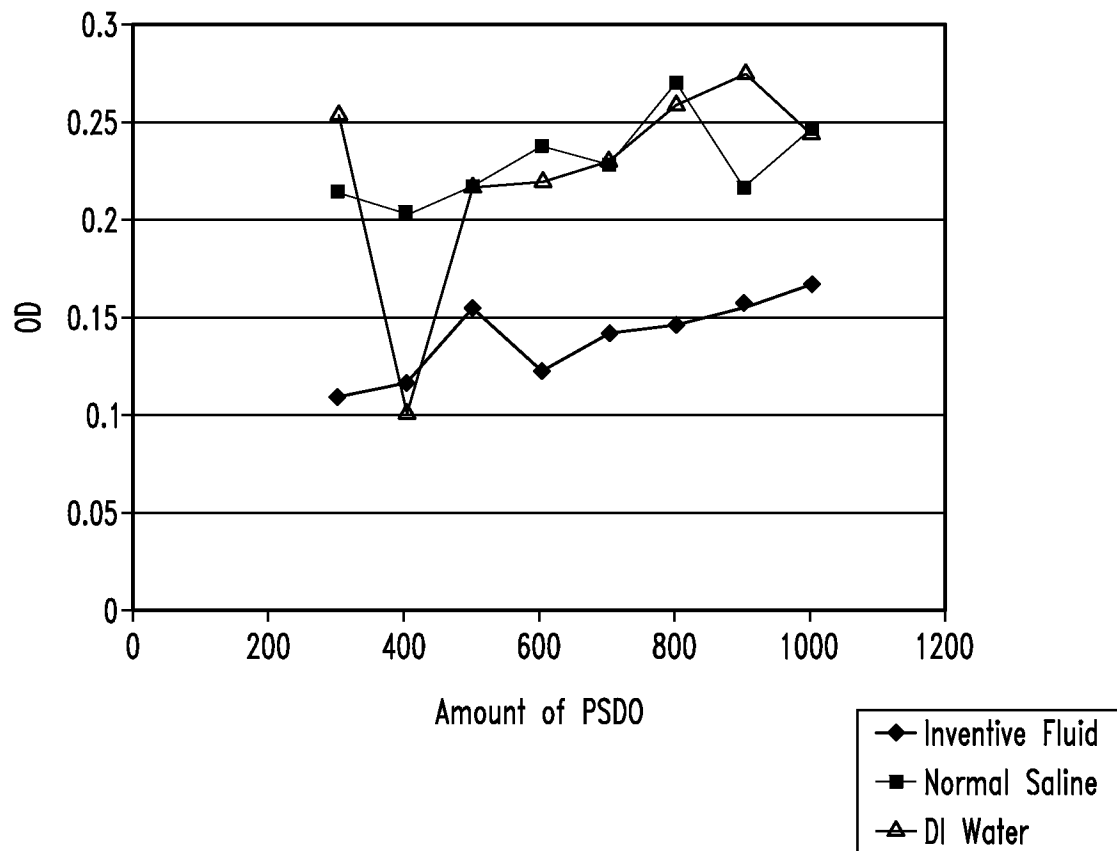


Fig. 38

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*Fig. 39*

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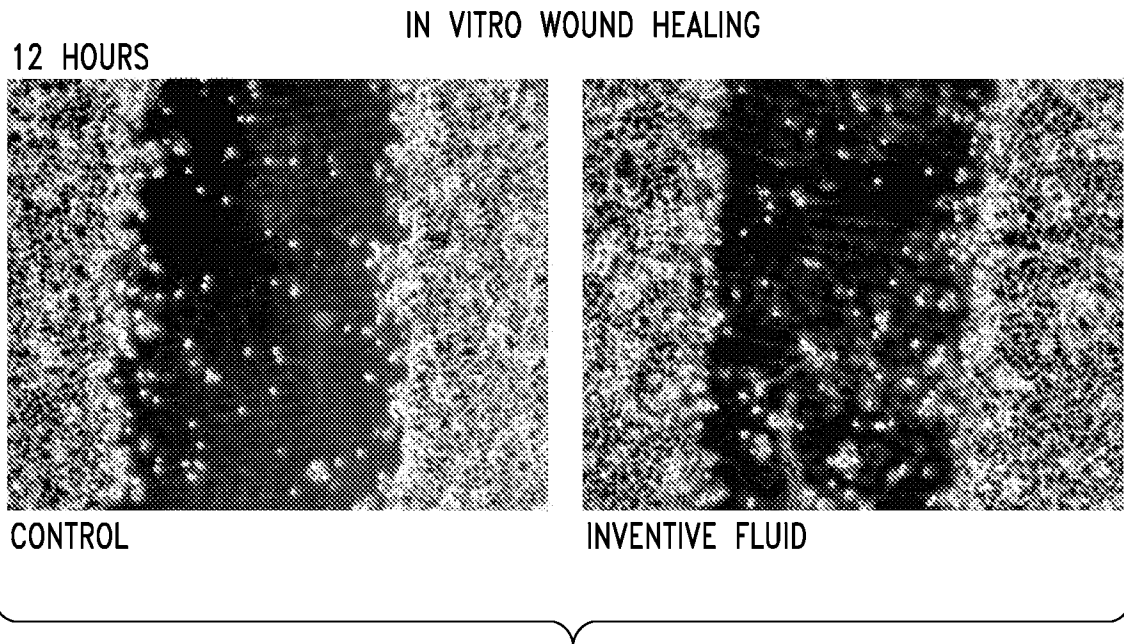


Fig. 40A

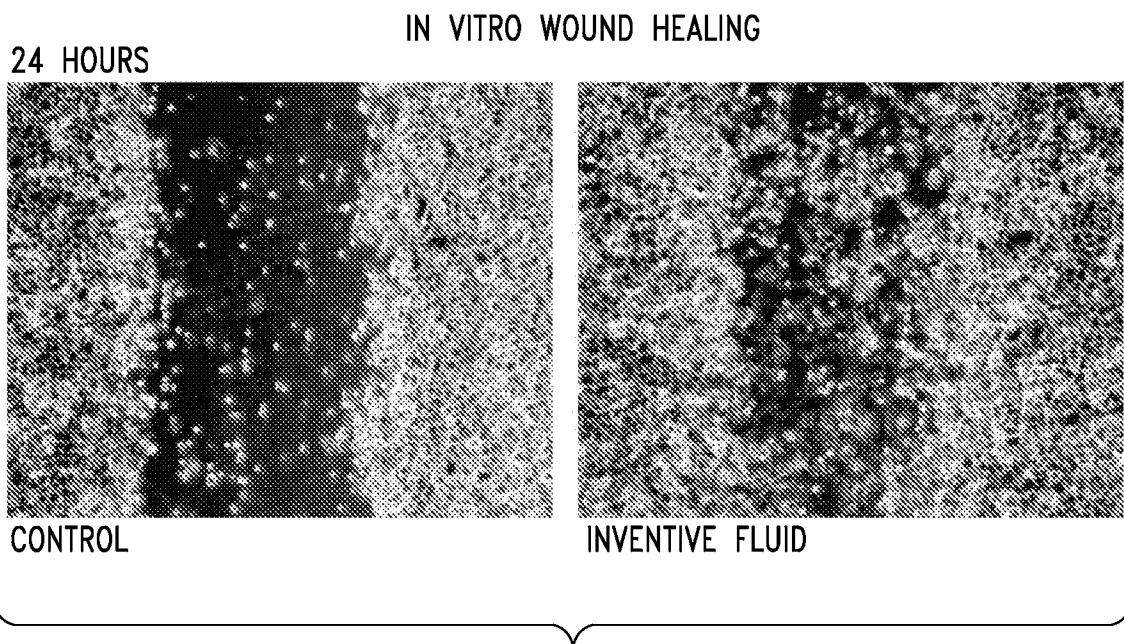
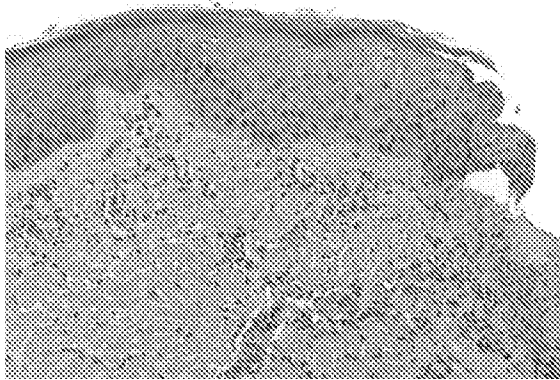


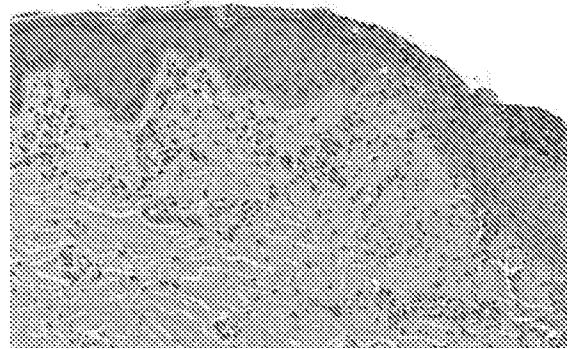
Fig. 40B

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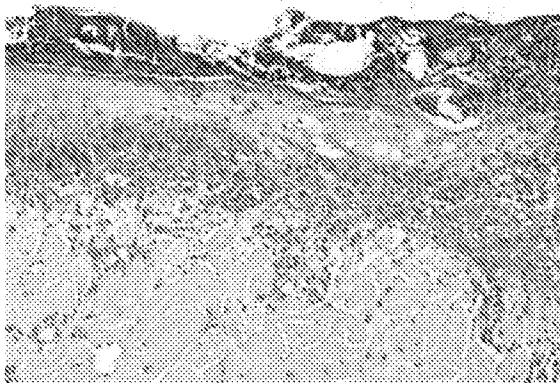
Control one day post wounding
showing epidermal/dermal thickening
and loss of contour

Fig. 41A



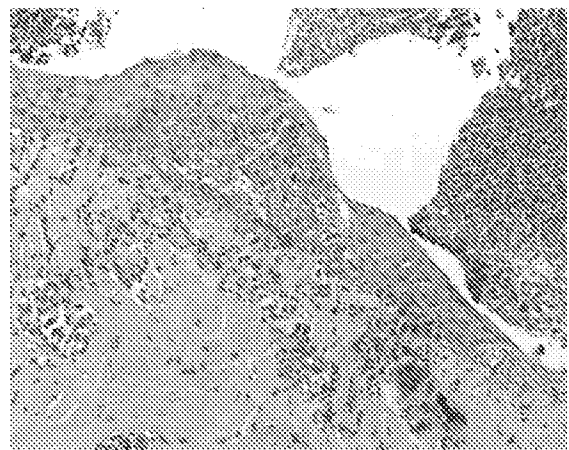
Experimental with inventive fluid one day post
wounding showing normal epidermal/dermal
thickness and normal contour

Fig. 41B



Control day four showing 600 micron
epidermal spur

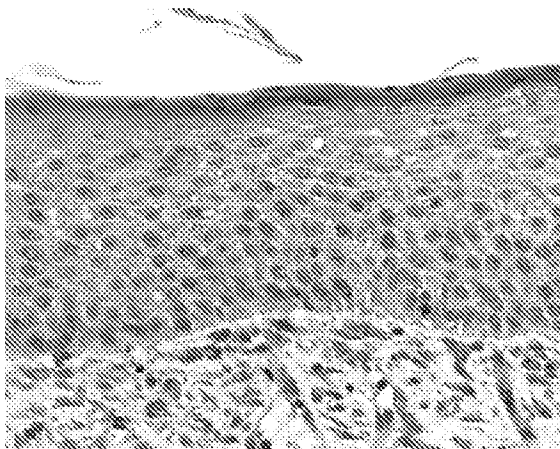
Fig. 41C



Experimental day four with inventive fluid
showing 1200 micron epidermal spur

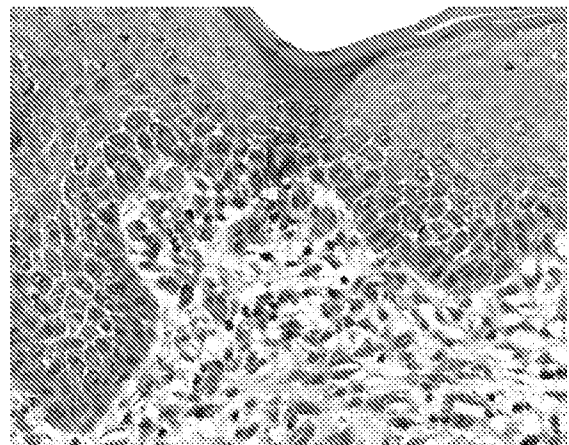
Fig. 41D

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Control day 16 showing less differentiated epidermis with loss of epidermal/dermal contour

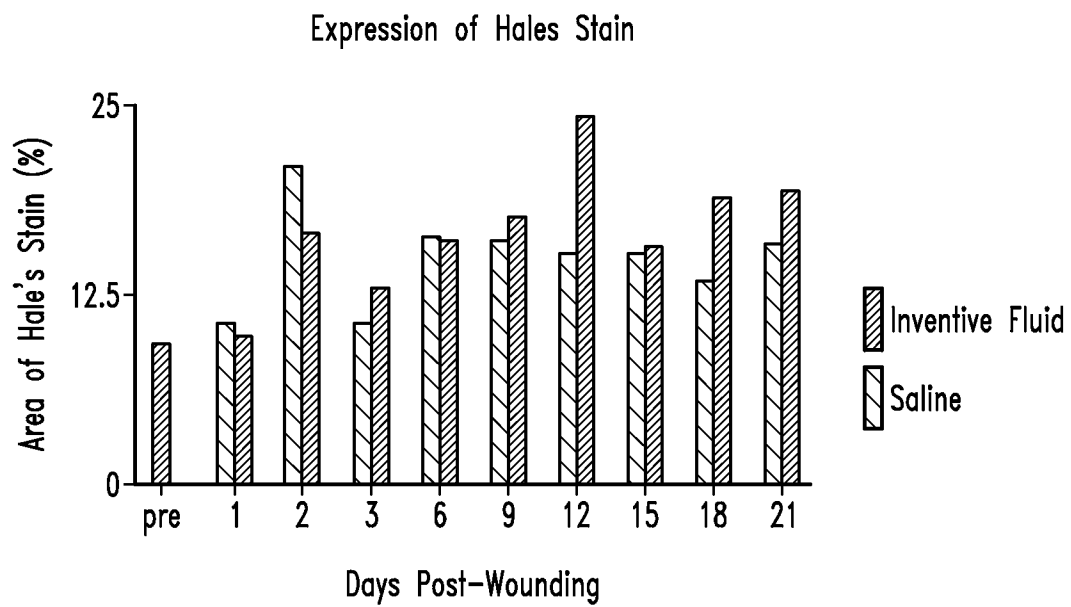
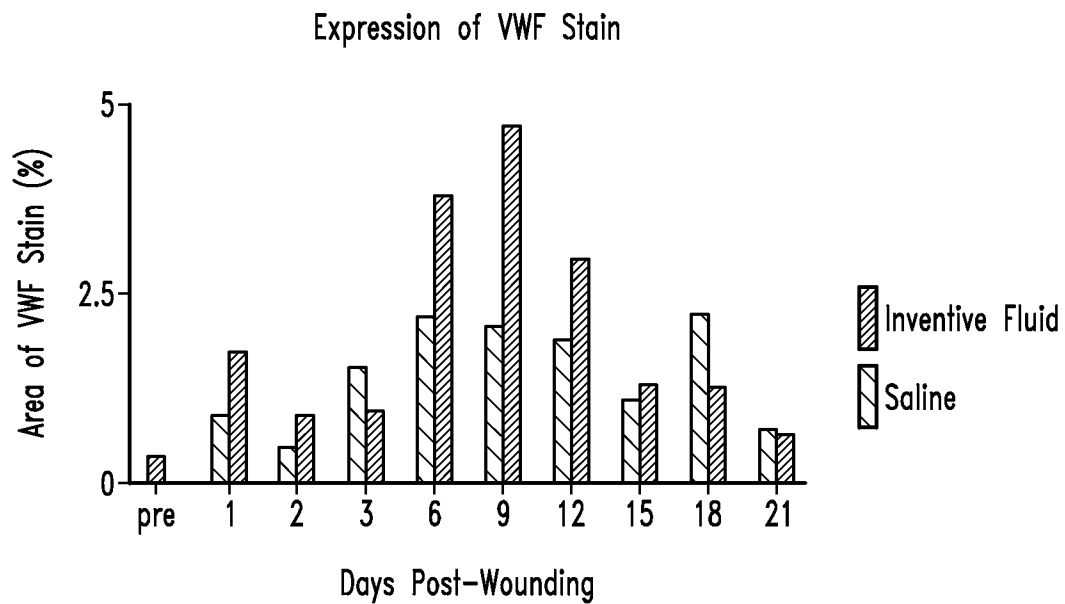
Fig. 41E



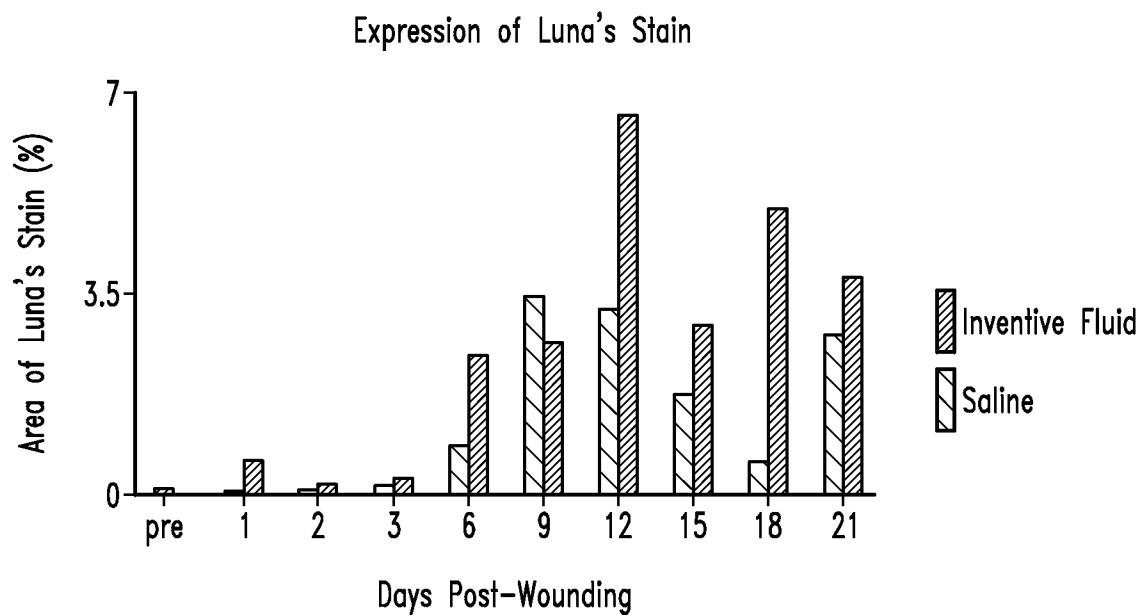
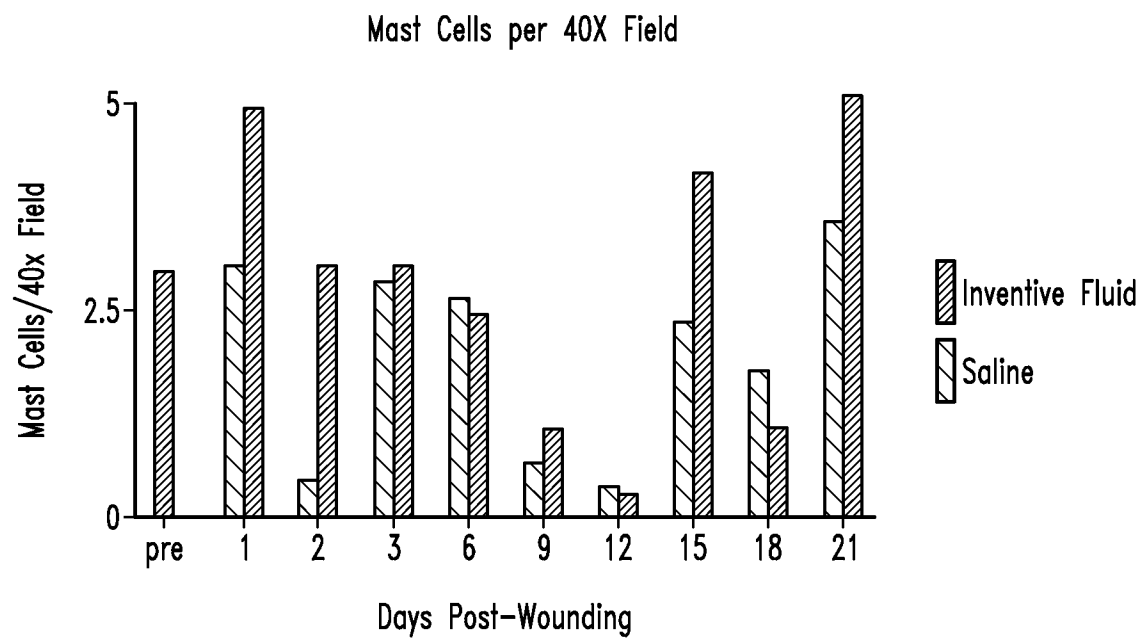
Experimental day 16 with inventive fluid showing more differentiated epidermis with more normal epidermal/dermal contour

Fig. 41F

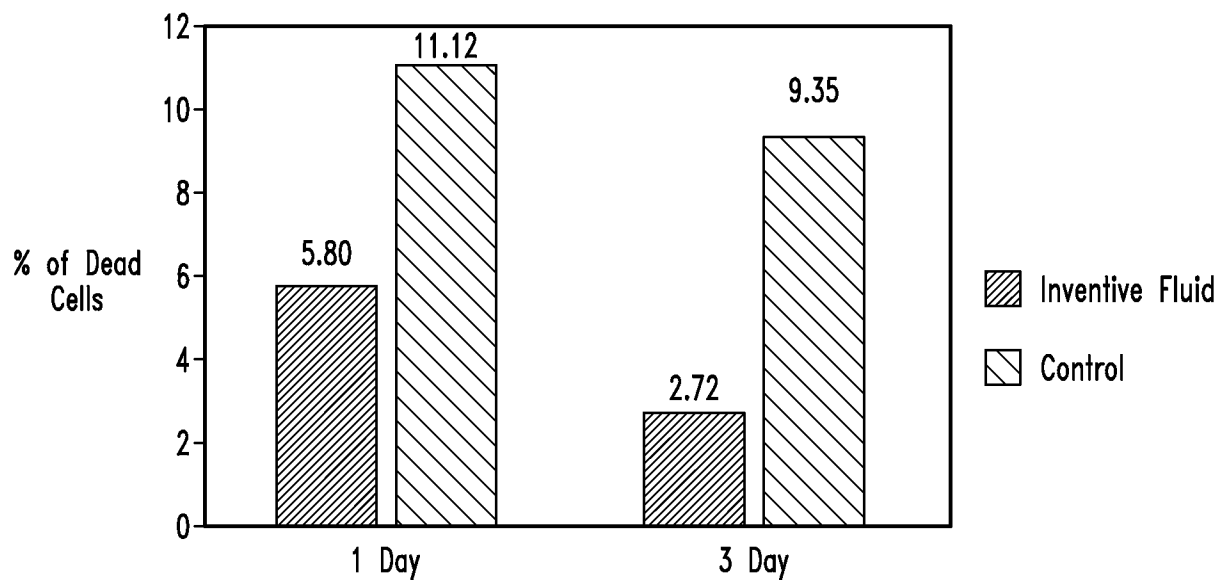
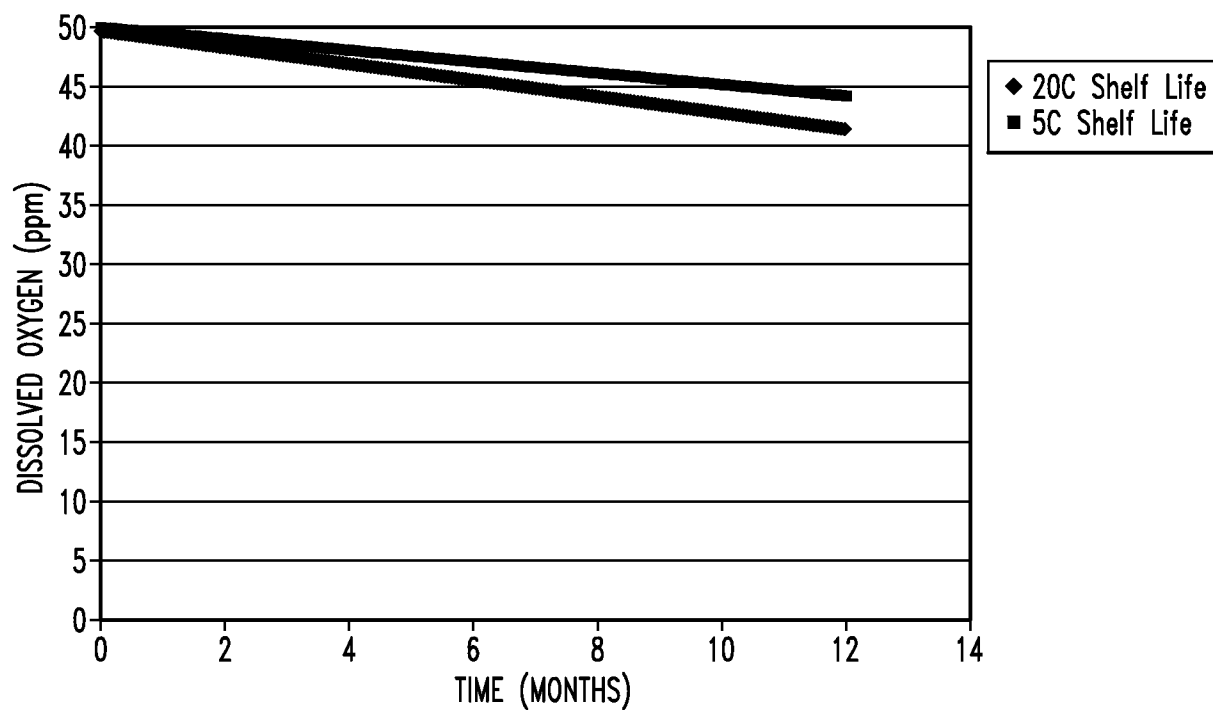
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*Fig. 42**Fig. 43*

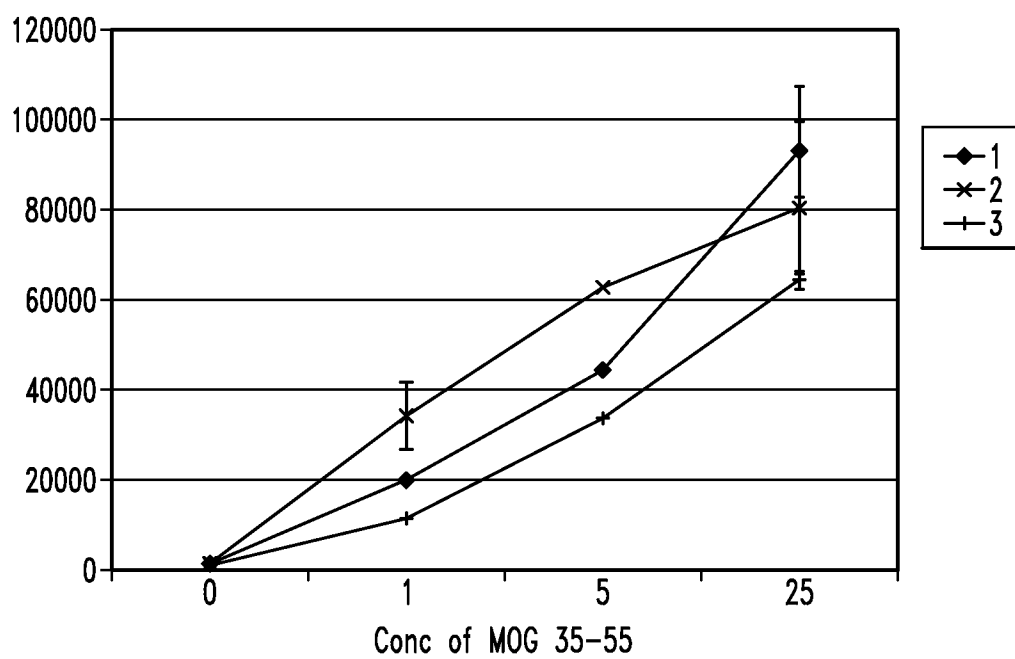
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*Fig. 44**Fig. 45*

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*Fig. 46**Fig. 47*

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*Fig. 48*

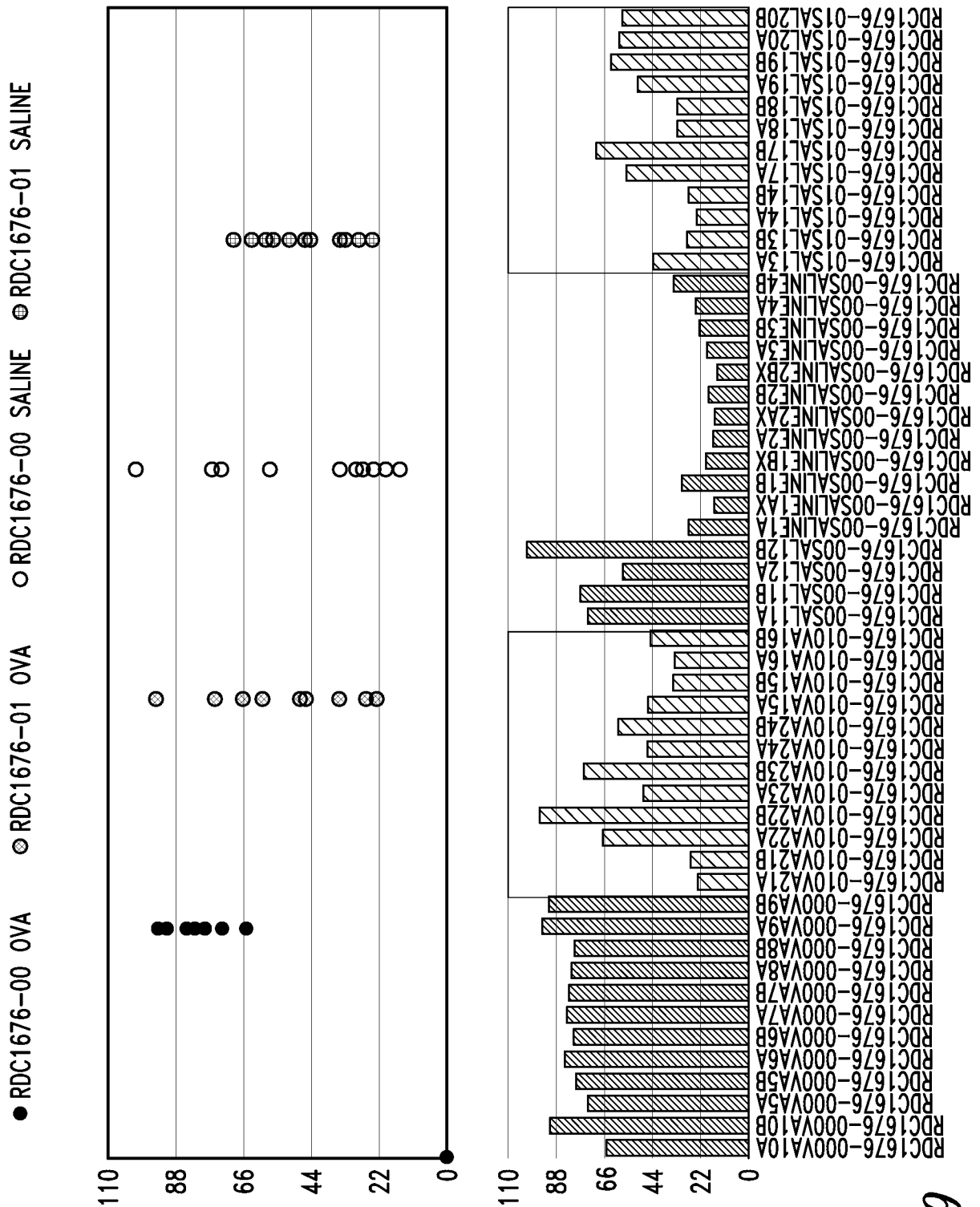


Fig. 49

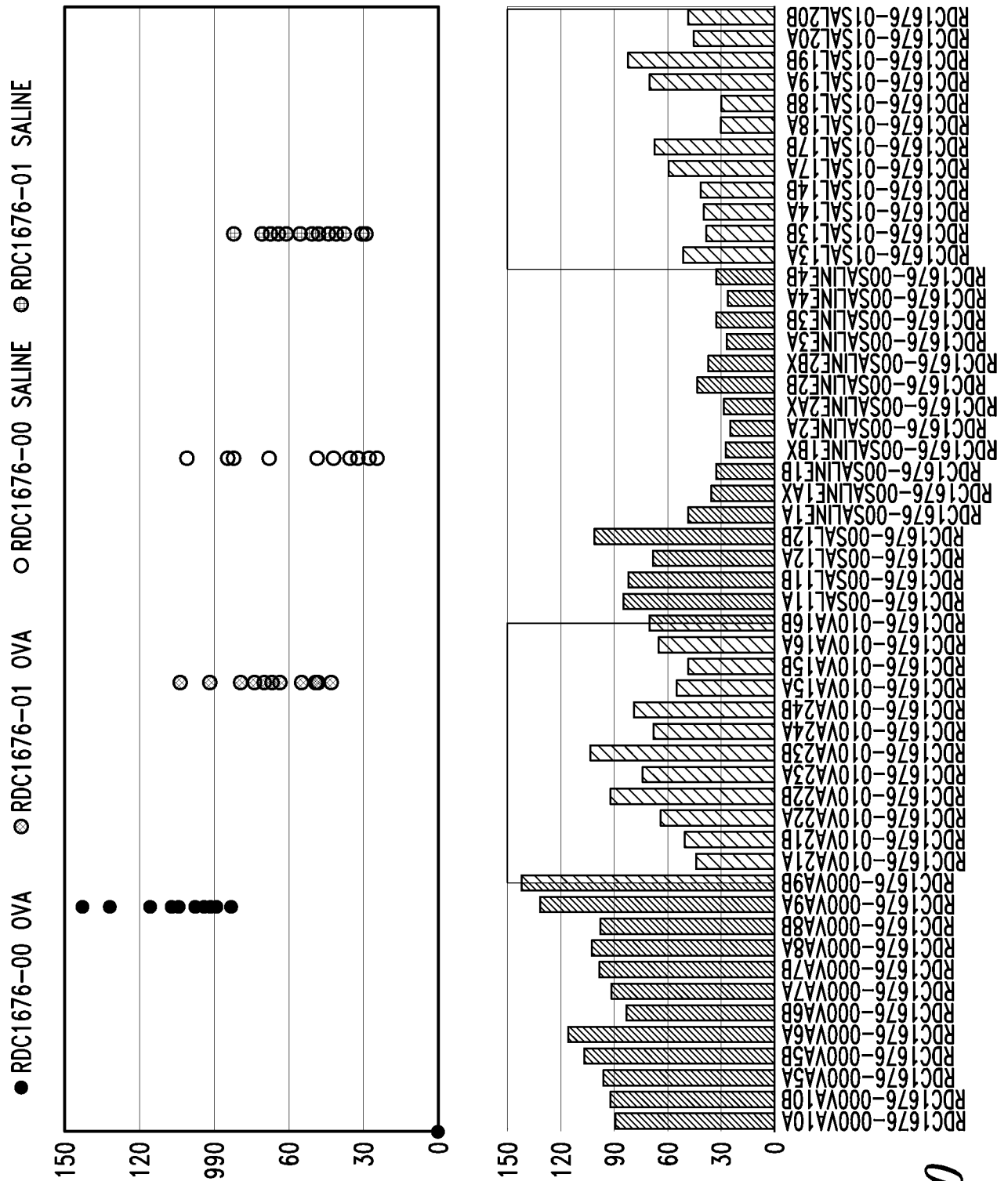


Fig. 50

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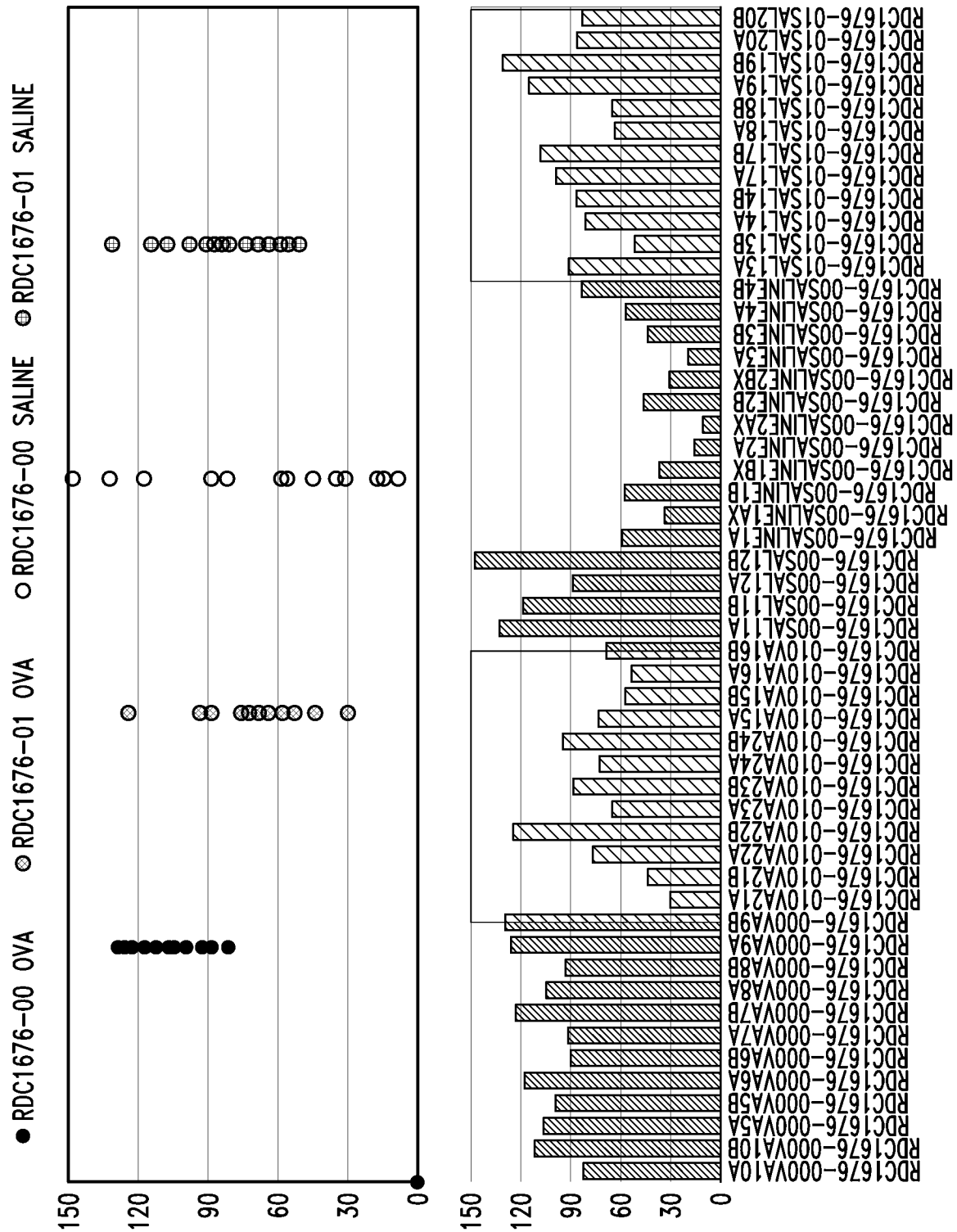


Fig. 51

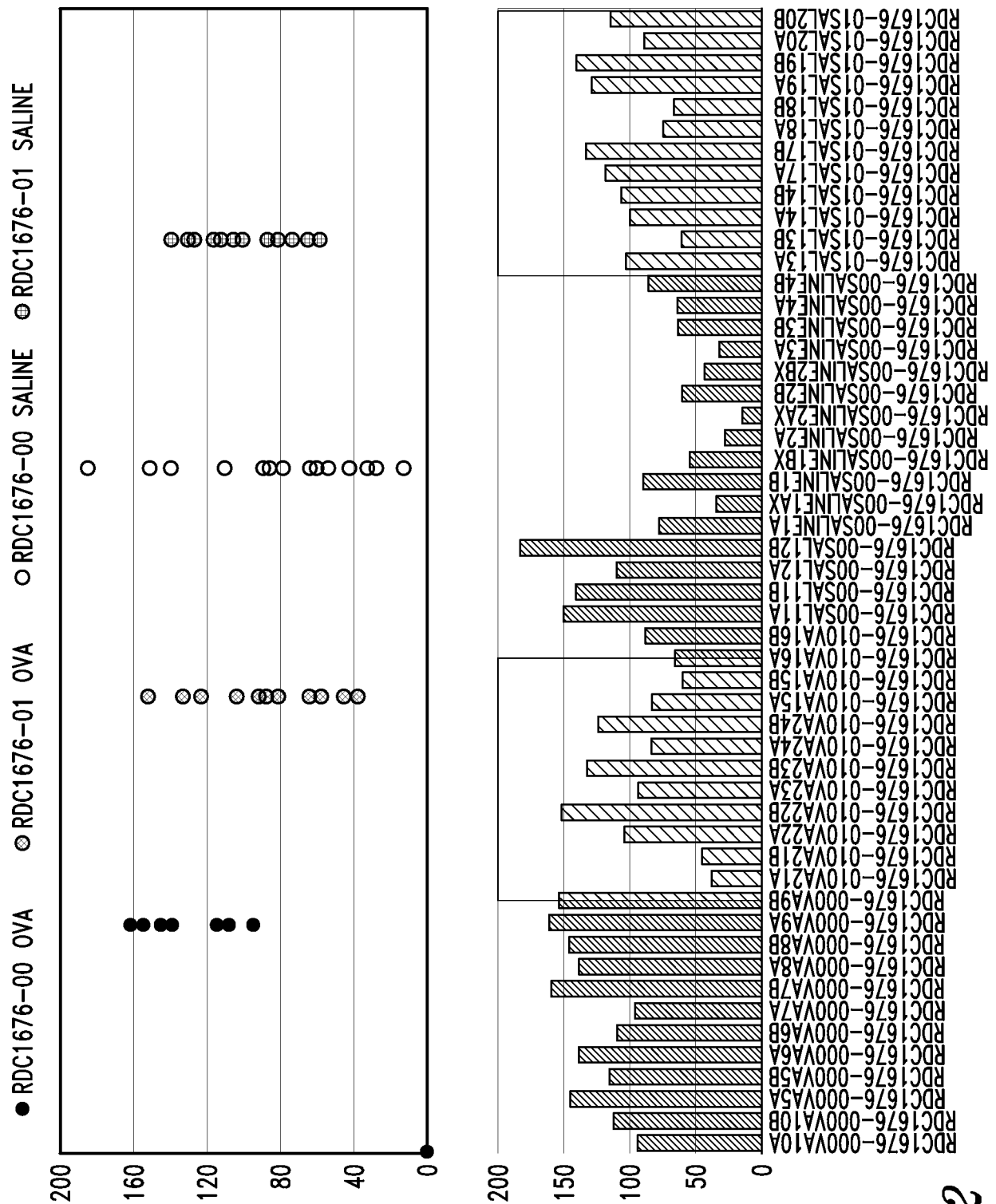


Fig. 52

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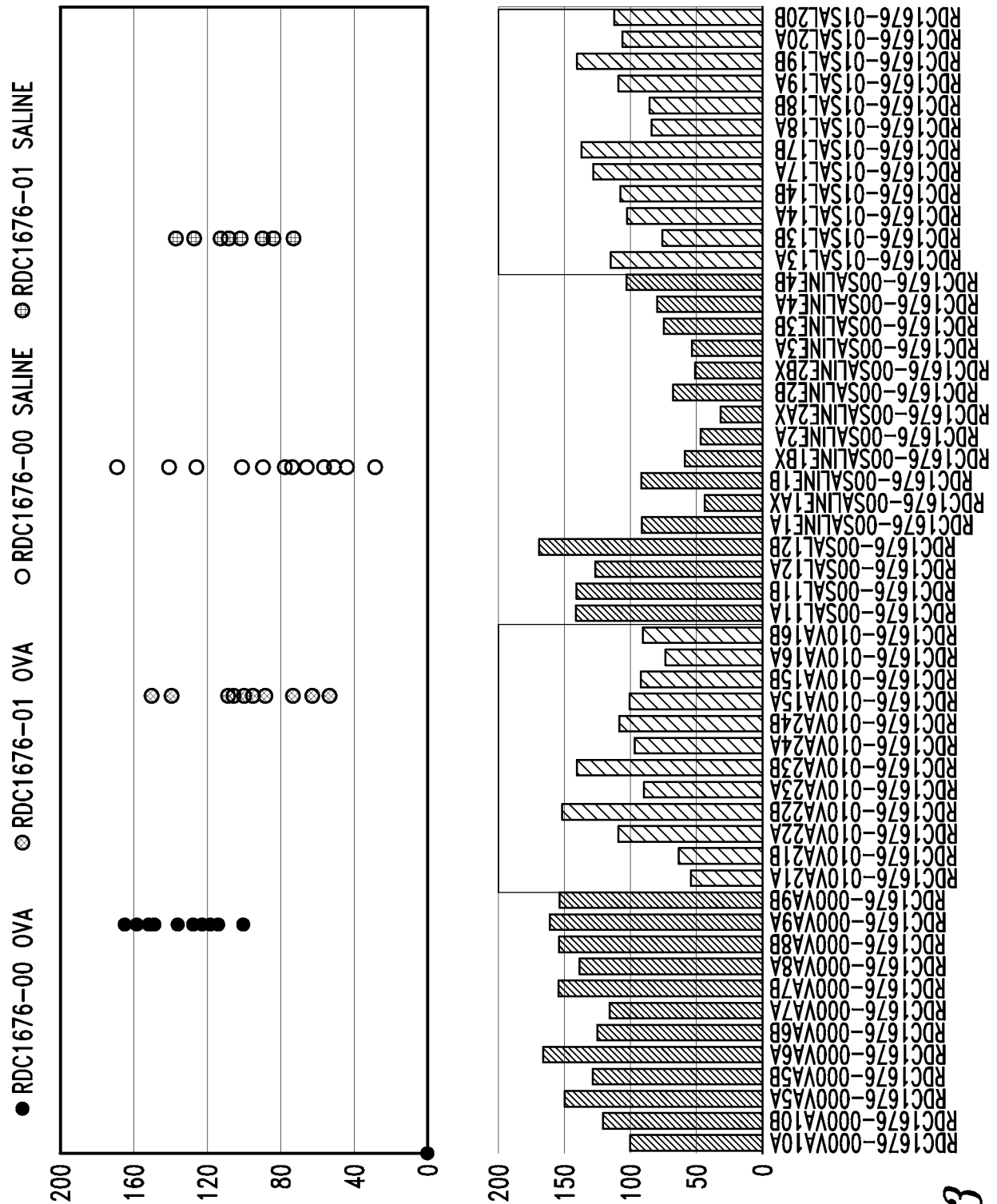


Fig. 53

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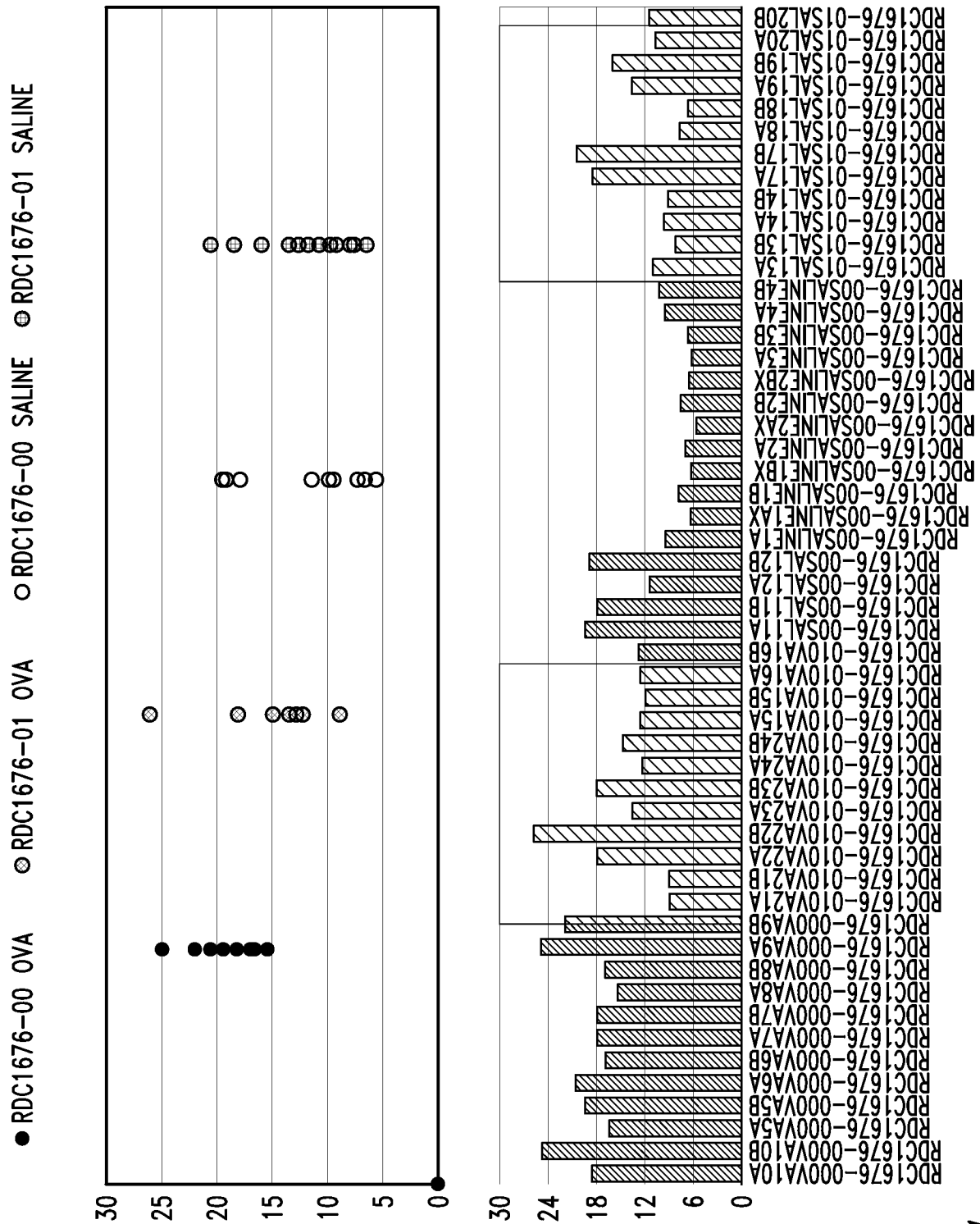


Fig. 54

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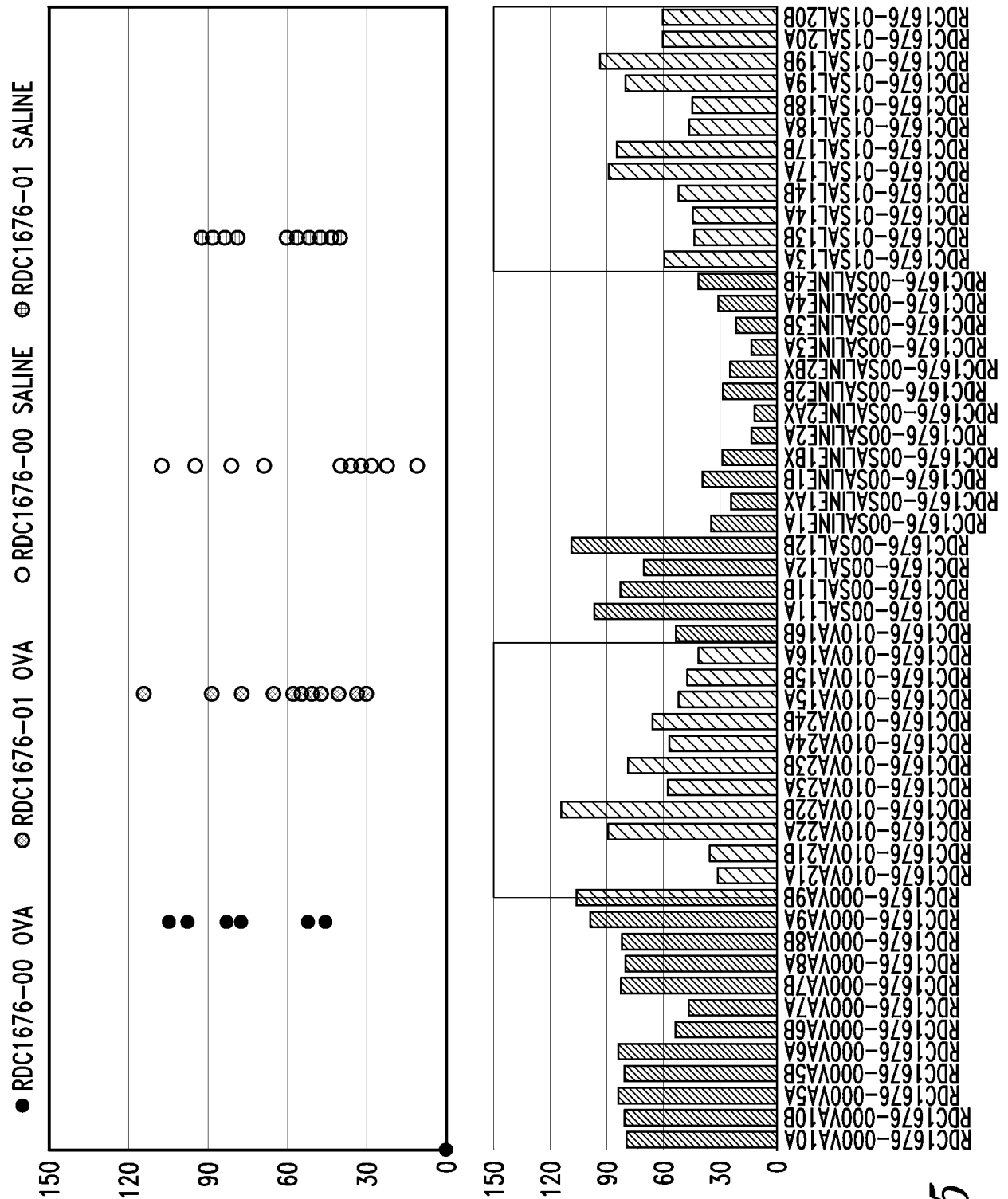


Fig. 55

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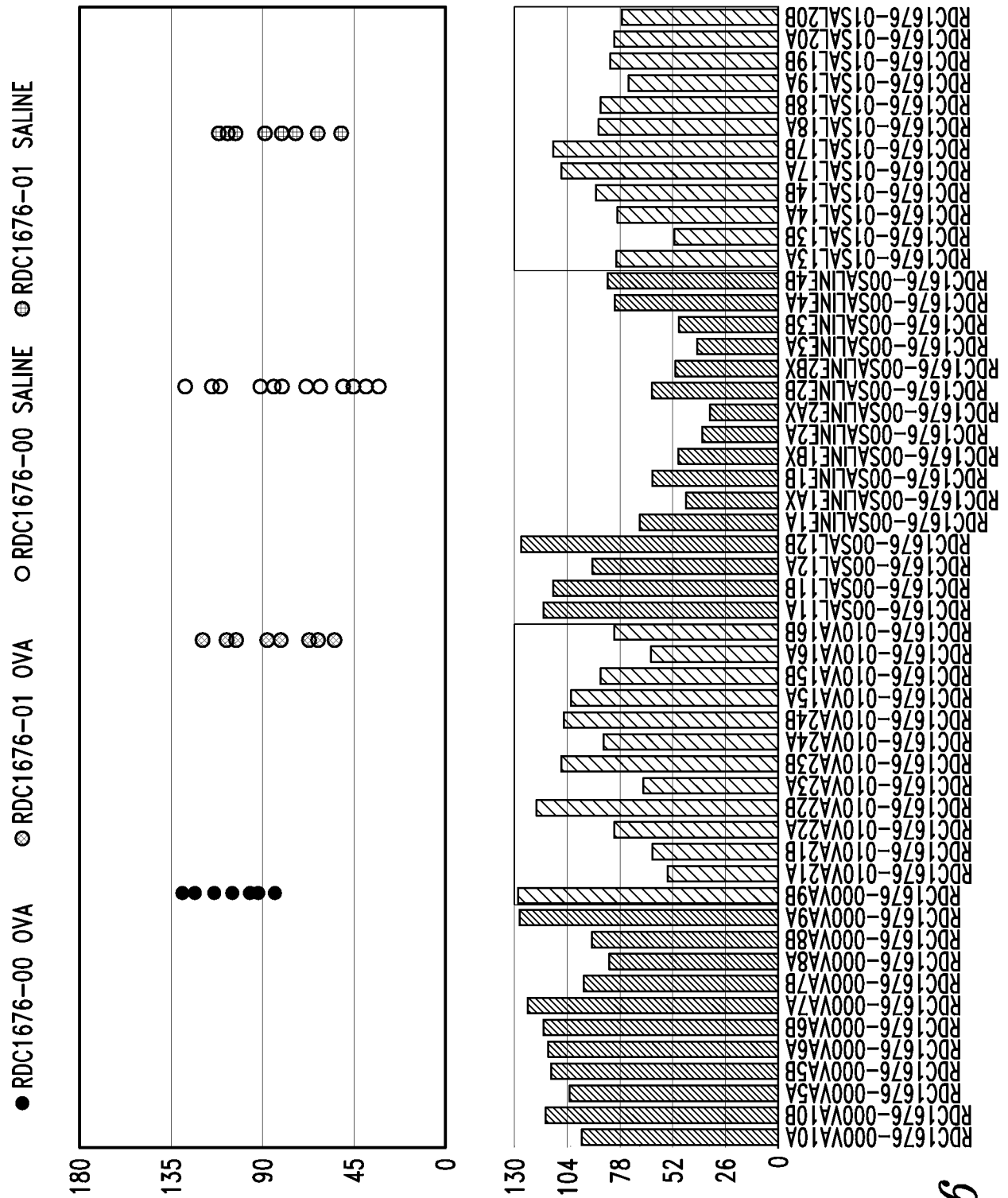


Fig. 56

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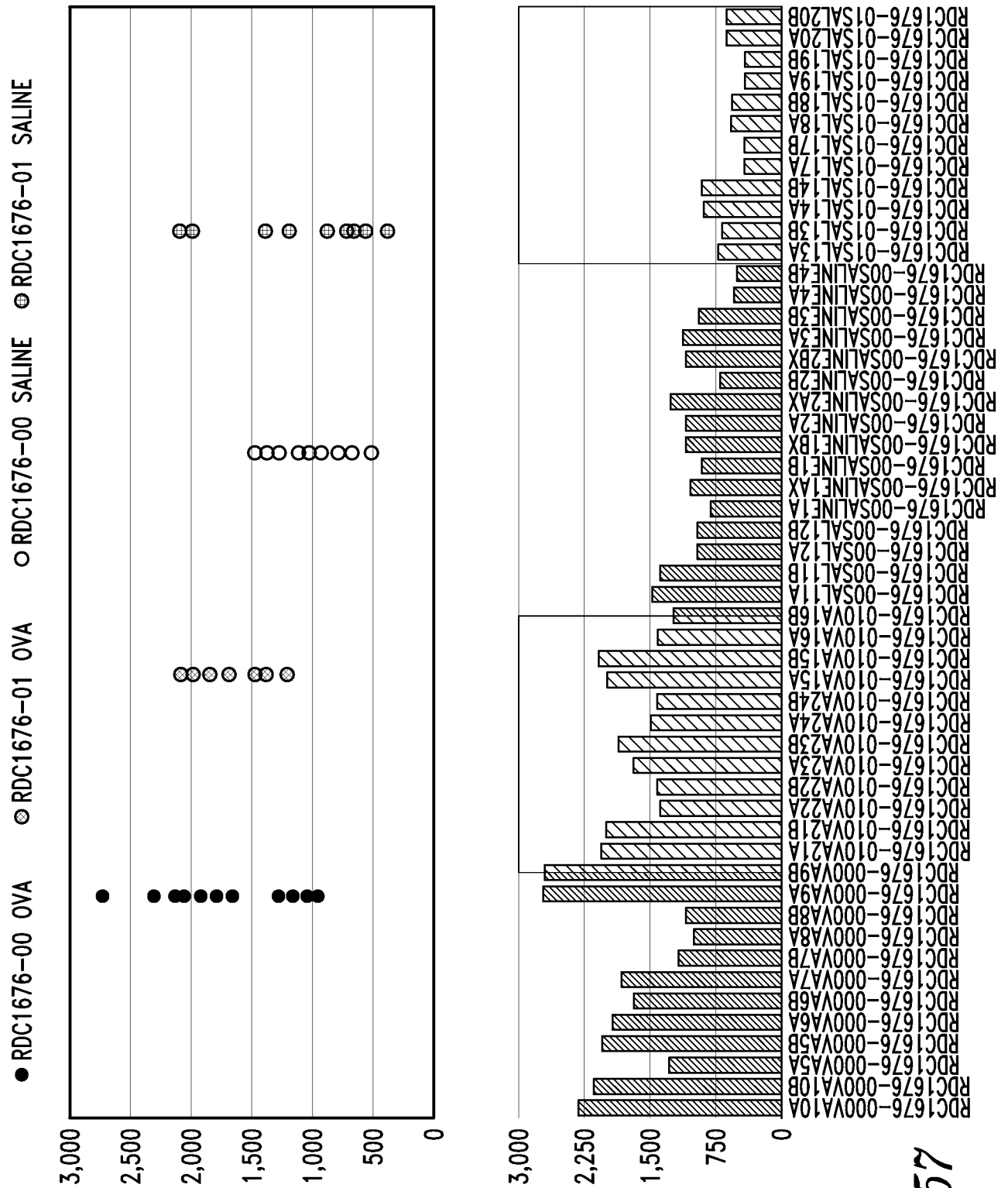


Fig. 57

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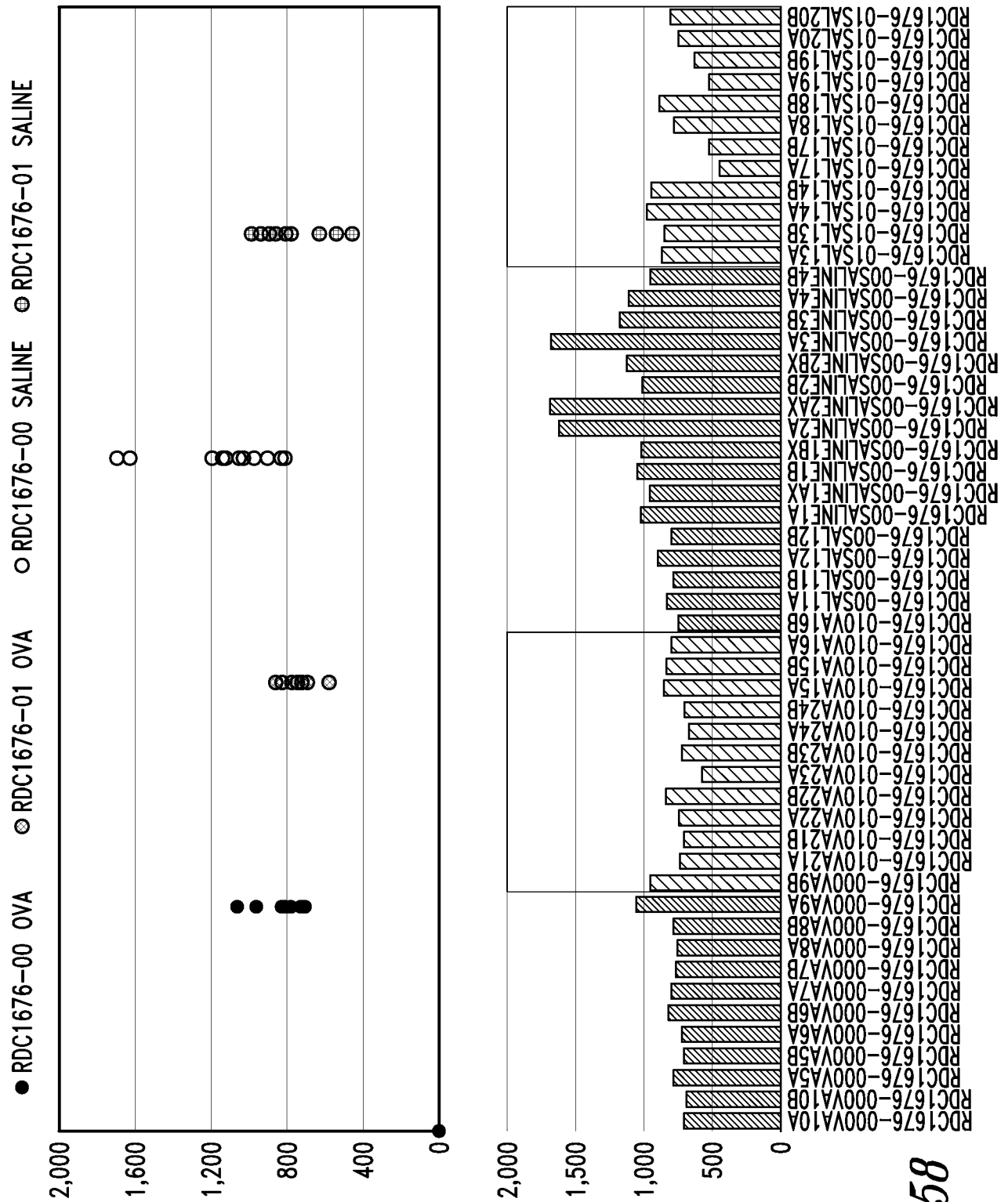


Fig. 58

● BAL1676-00 OVA ⊙ BAL1676-01 OVA ○ BAL1676-00 SALINE ⊕ BAL1676-01 SALINE

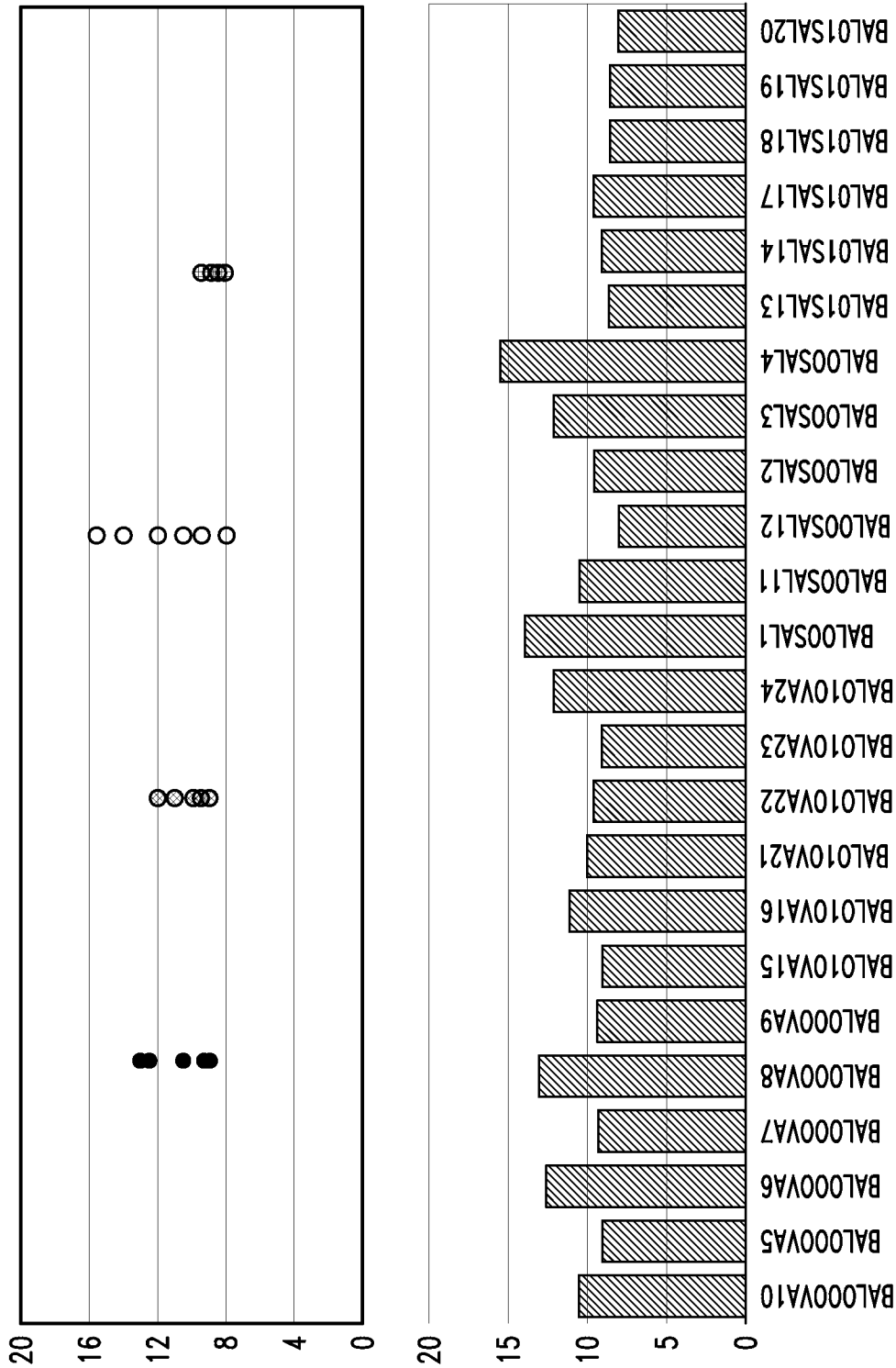


Fig. 59

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● BAL1676-00 OVA ⊙ BAL1676-01 OVA ○ BAL1676-00 SALINE ⊕ BAL1676-01 SALINE

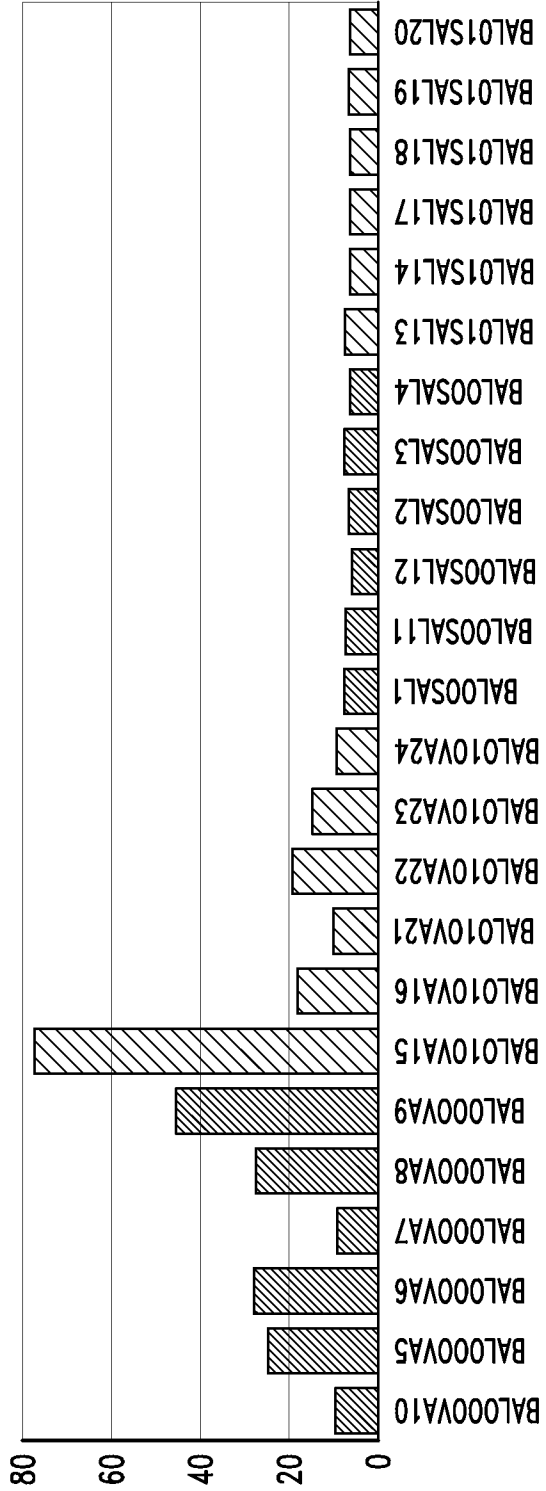
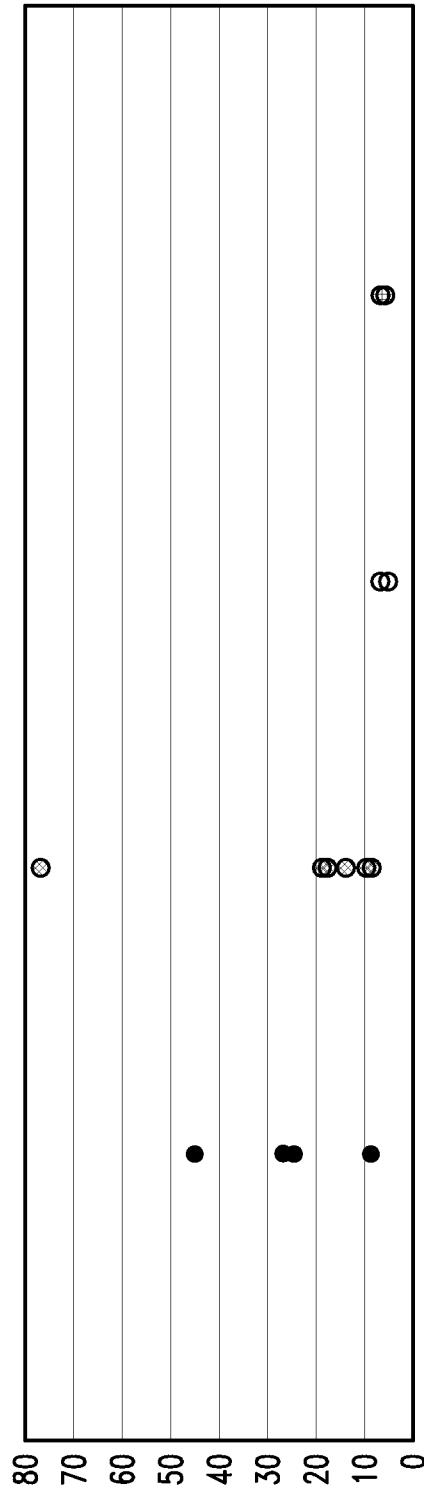


Fig. 60

● BAL1676-00 OVA ⊙ BAL1676-01 OVA ○ BAL1676-00 SALINE ⊕ BAL1676-01 SALINE

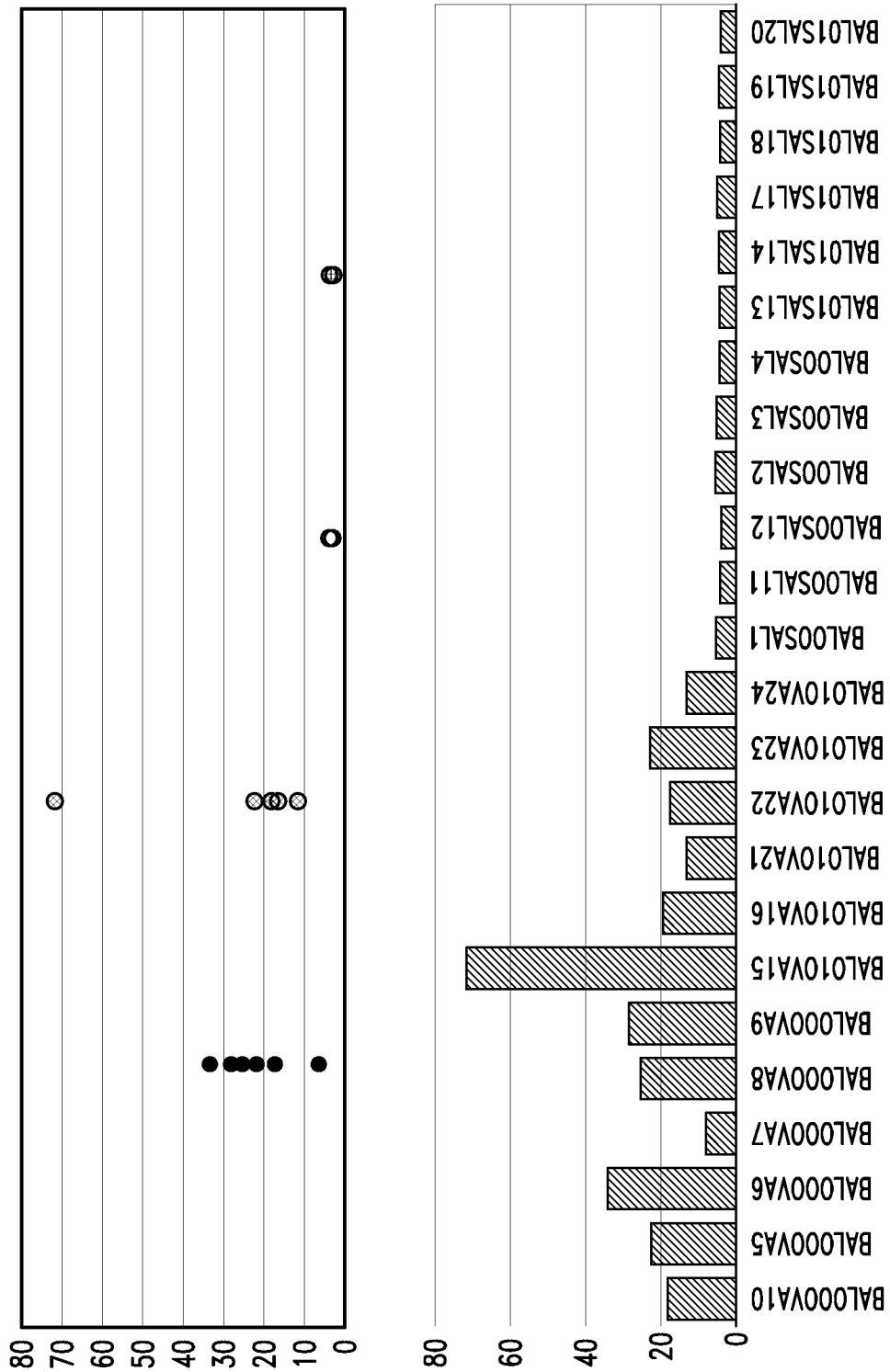


Fig. 61

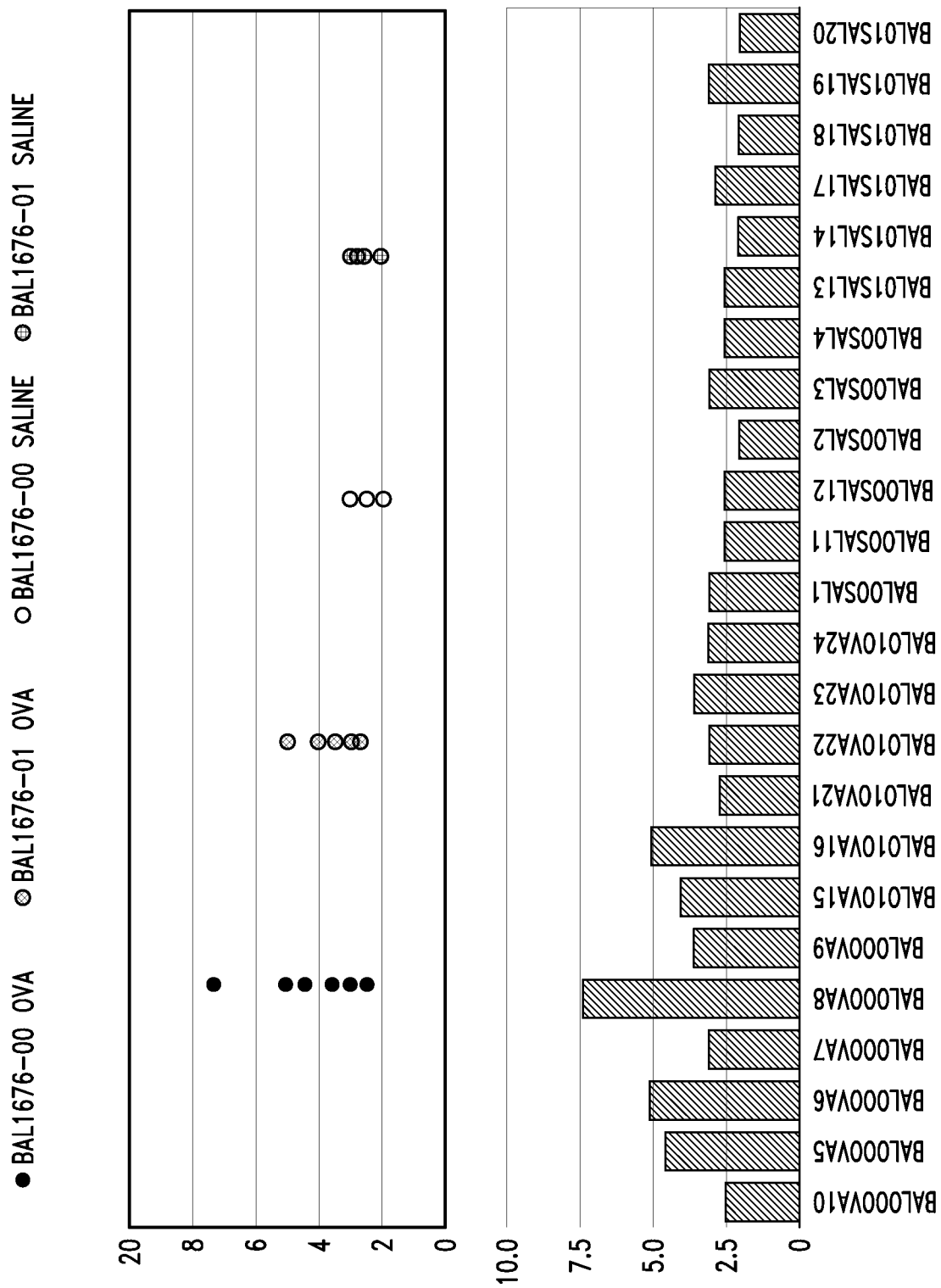


Fig. 62

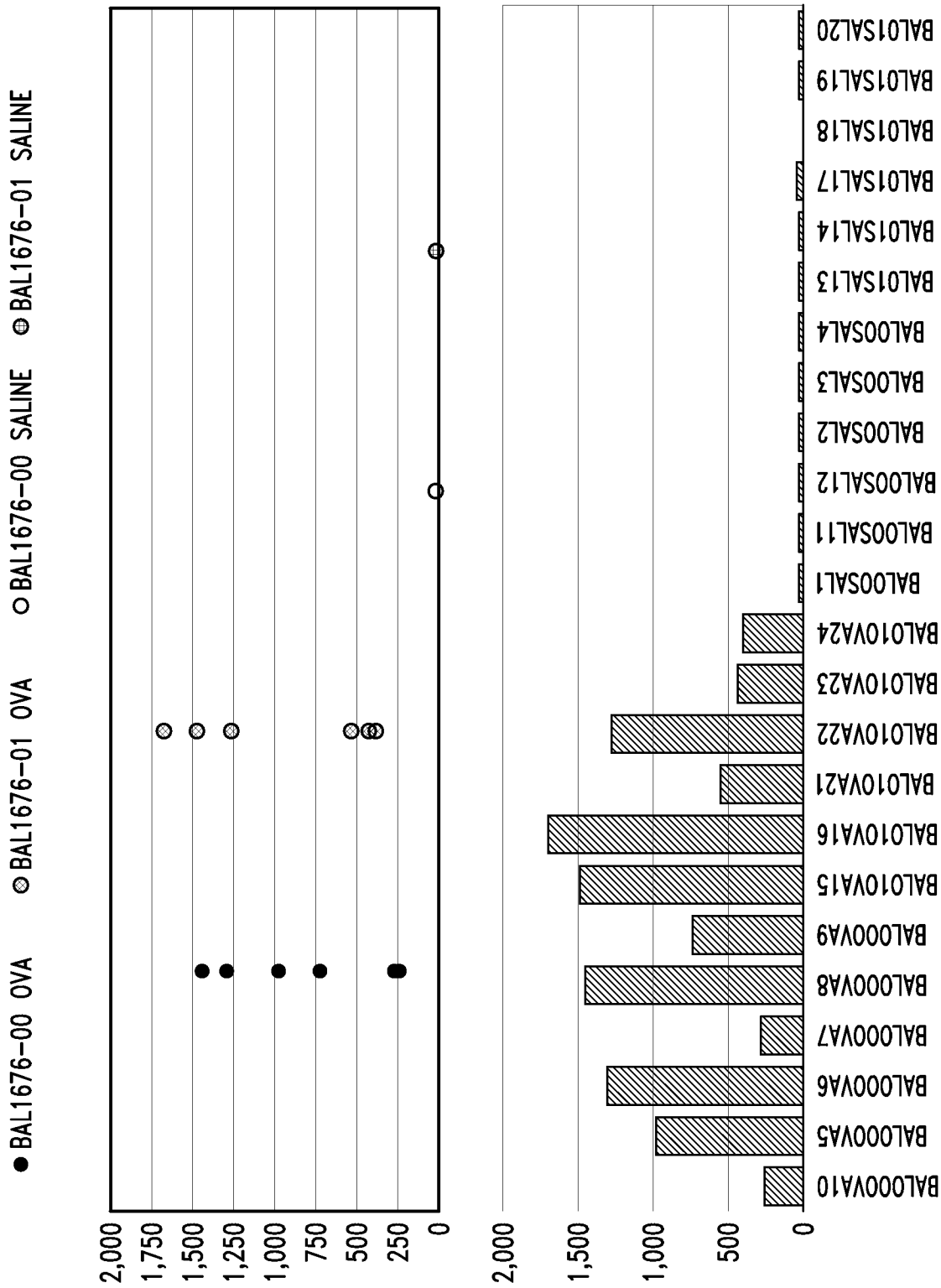


Fig. 63

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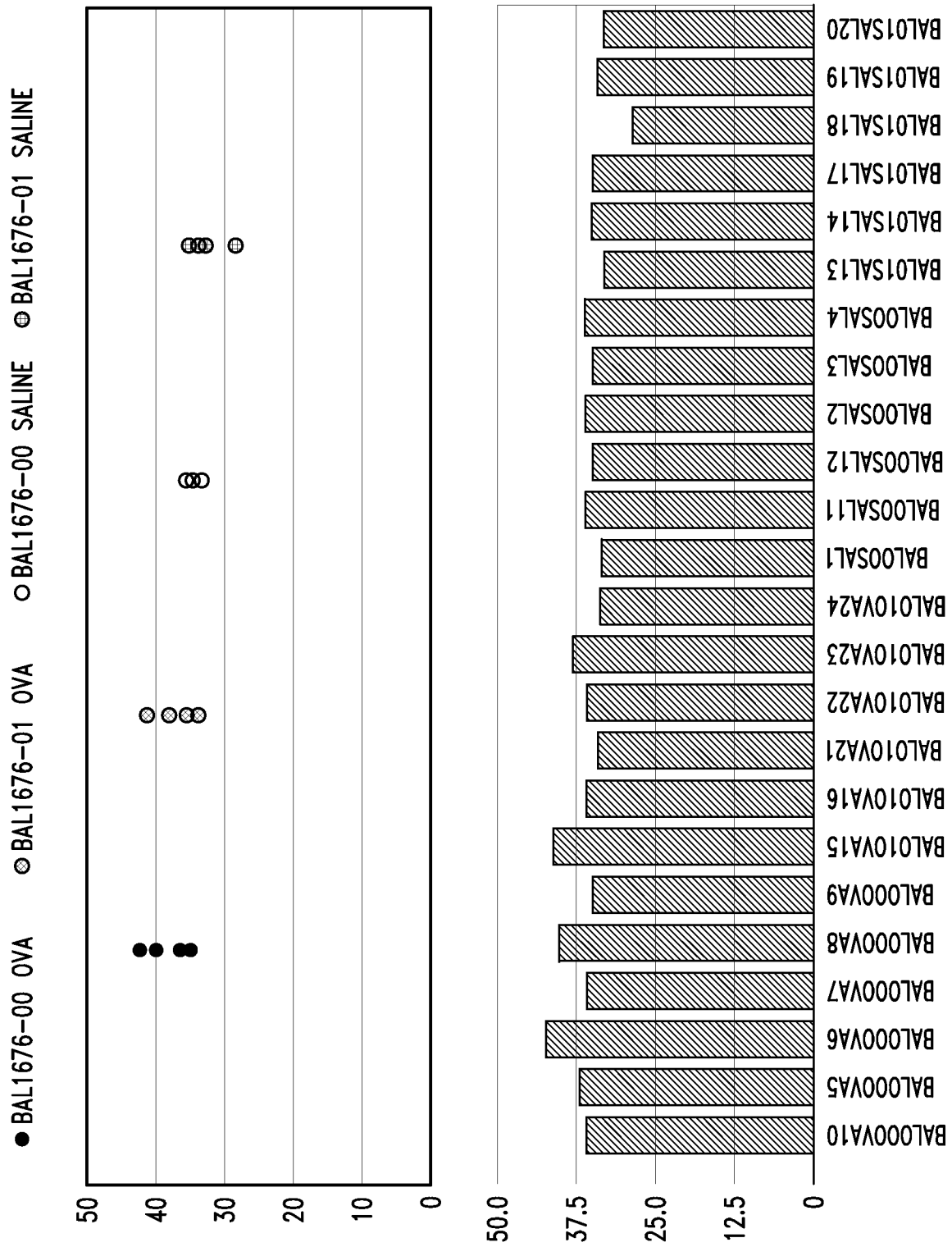


Fig. 64

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● BAL1676-00 OVA ⊙ BAL1676-01 OVA ○ BAL1676-00 SALINE ⊕ BAL1676-01 SALINE

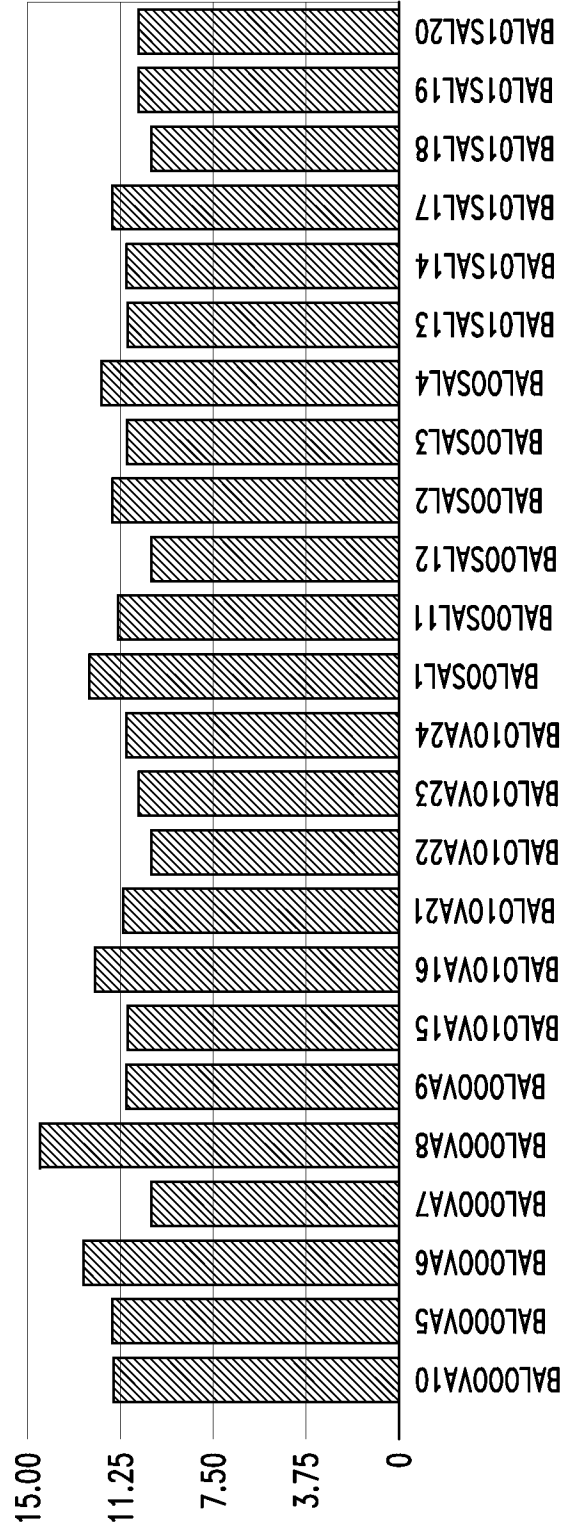
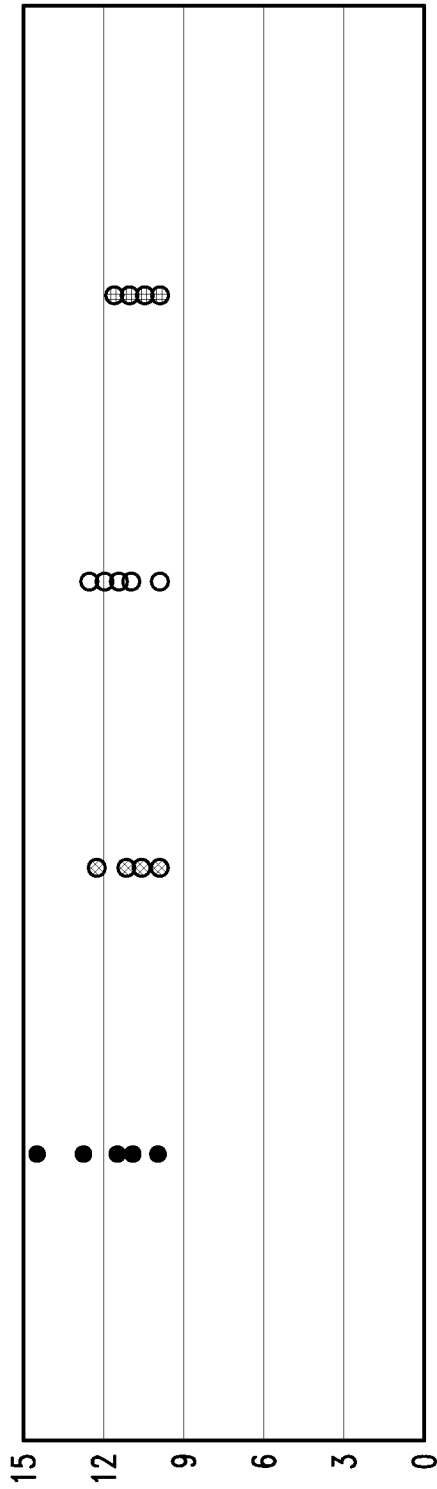


Fig. 65

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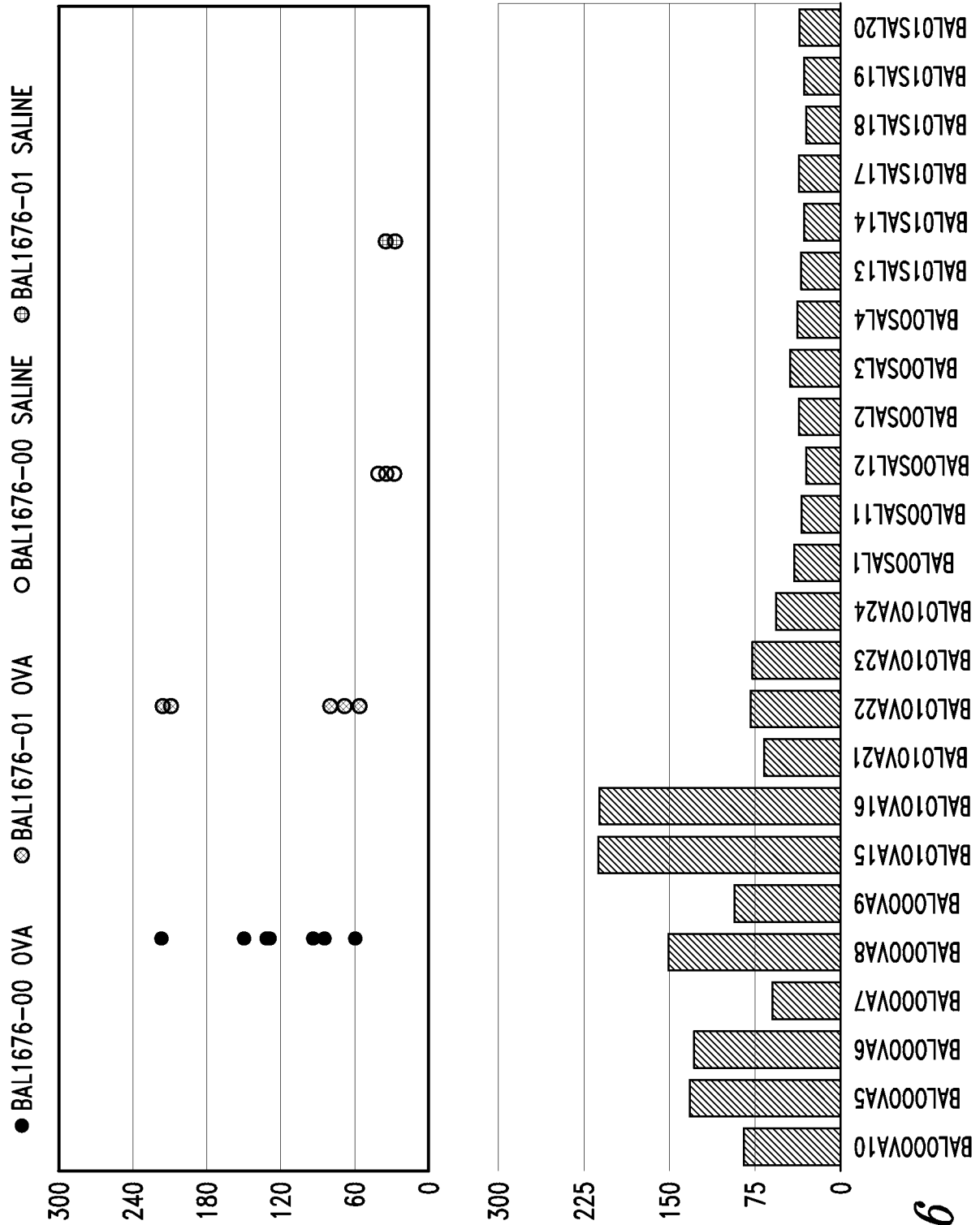


Fig. 66

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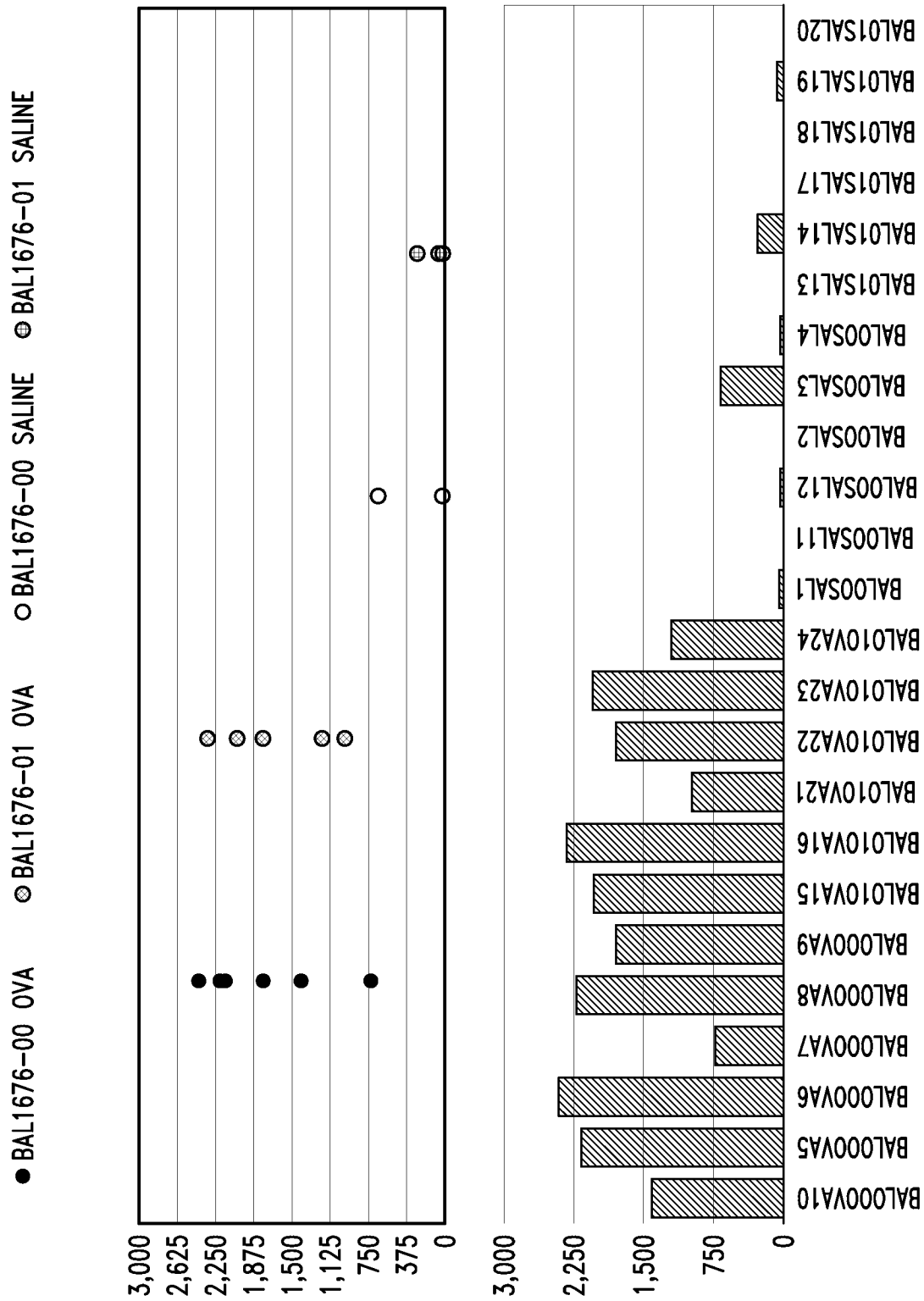


Fig. 67

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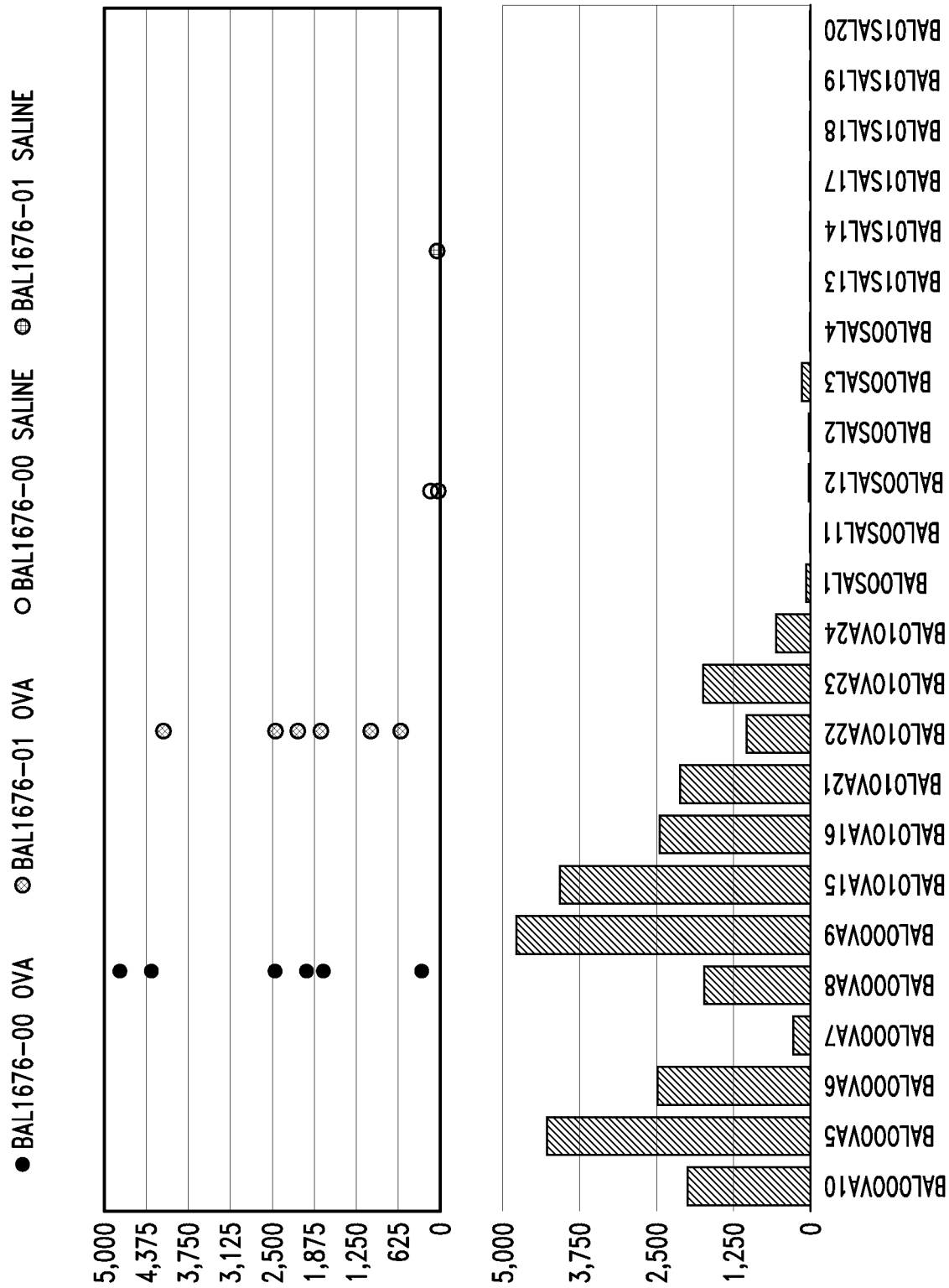


Fig. 68

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Step Data Setup				Assay Step Lists			
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☒ Def Assoc.	900	1000	Assoc.	1	☐ Col 1	☒ Col 1	☐ Def Regeneration
☐ Def Dissoc.	900	1000	Dissoc.		☐ Col 1	☒ Col 1	☐ baseline1
☐ Def Baseline No Fl...	600	0	Baseline		☐ Col 1	☒ Col 2	☐ loading
☐ Def Loading No Flow	900	0	Loading		☐ Col 1	☒ Col 3	☐ baseline2
☐ Def Activation	600	1000	Activation		☐ Col 1	☒ Col 4	☐ bradykinin
☐ Def Quenching	600	1000	Quenching		☐ Col 1	☒ Col 3	☐ dissociation
☐ Def Regeneration	30	1000	Regeneration				
☐ baseline1	30	1000	Baseline				
☐ loading	400	1000	Loading				
☐ baseline2	60	1000	Baseline				
☐ bradykinin	600	1000	Assoc.				
☐ dissociation	900	1000	Dissoc.				
				Sample plate set-up			
				Column 1: saline			
				Column 2: receptor B2 (50ug/mL) in saline			
				Column 3: A-D revsaline; E-H saline			
				Column 4: A-D Bradykinin at 1.25mM, 125uM 12.5uM in revsaline			
				E-H Bradykinin at 1.25mM, 125uM, 12.5uM in saline			
☒ Add ☐ Remove Time in (sec), flow rate in (rpm)				☒ Move			

Fig. 69

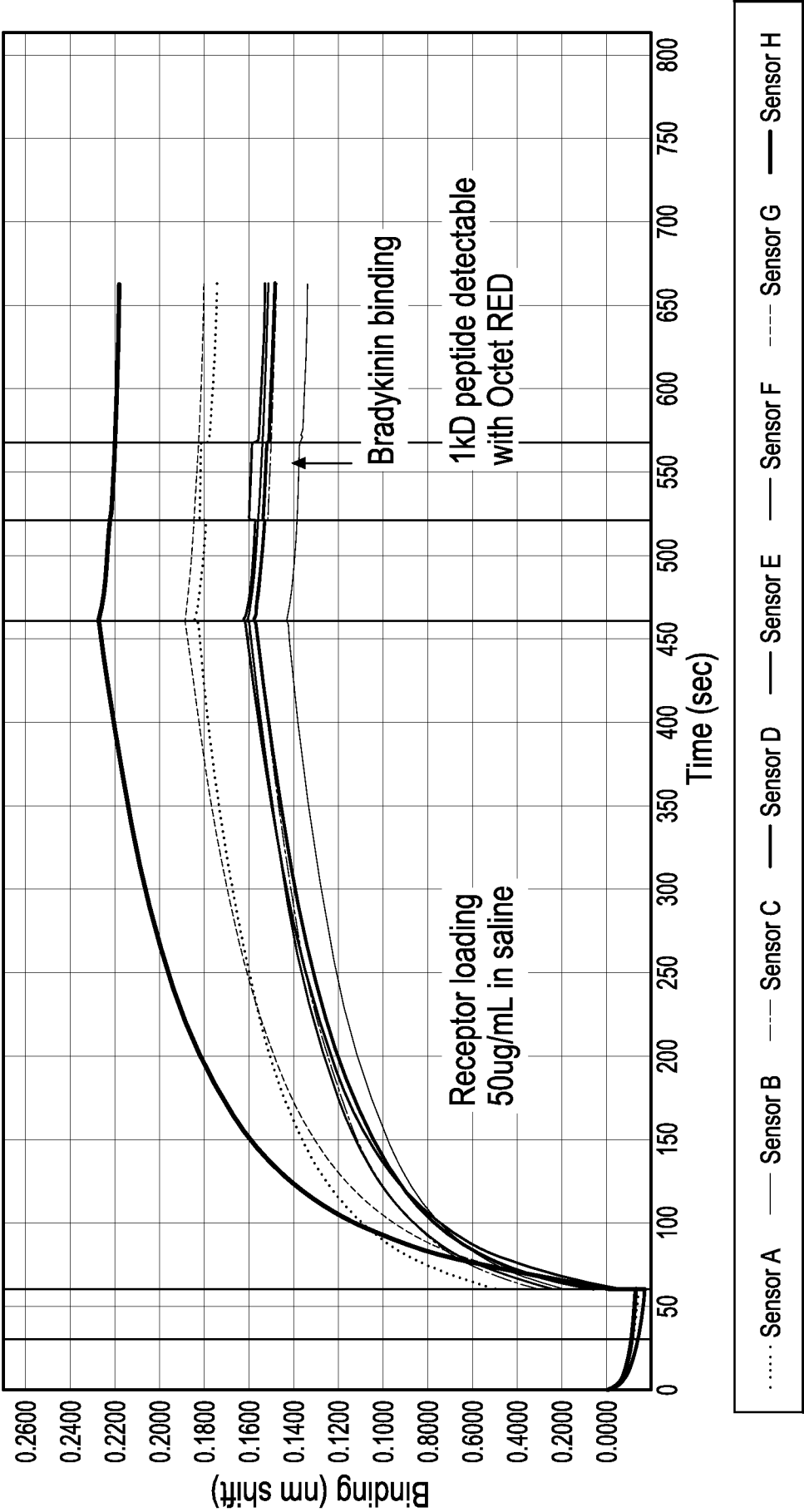
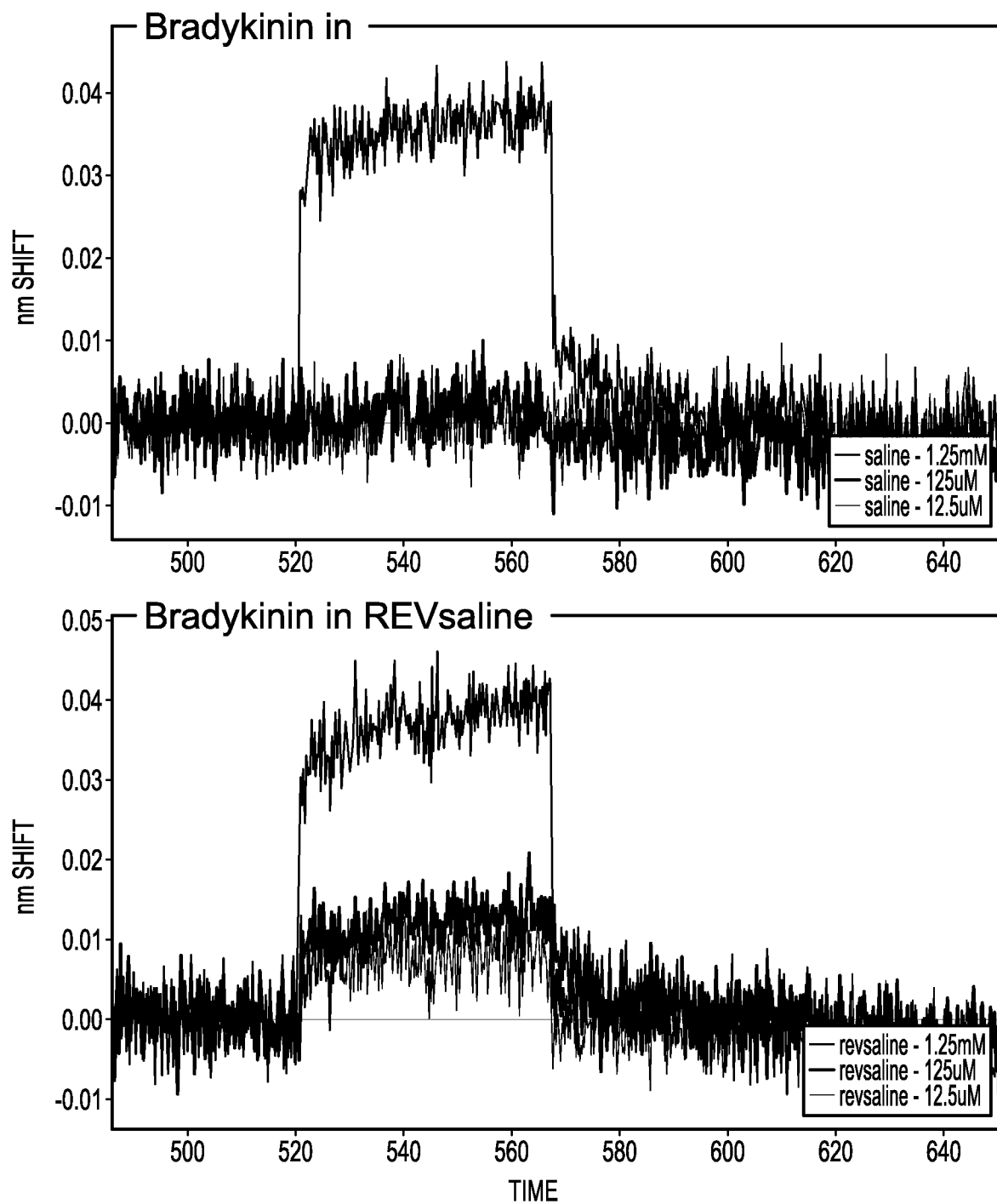
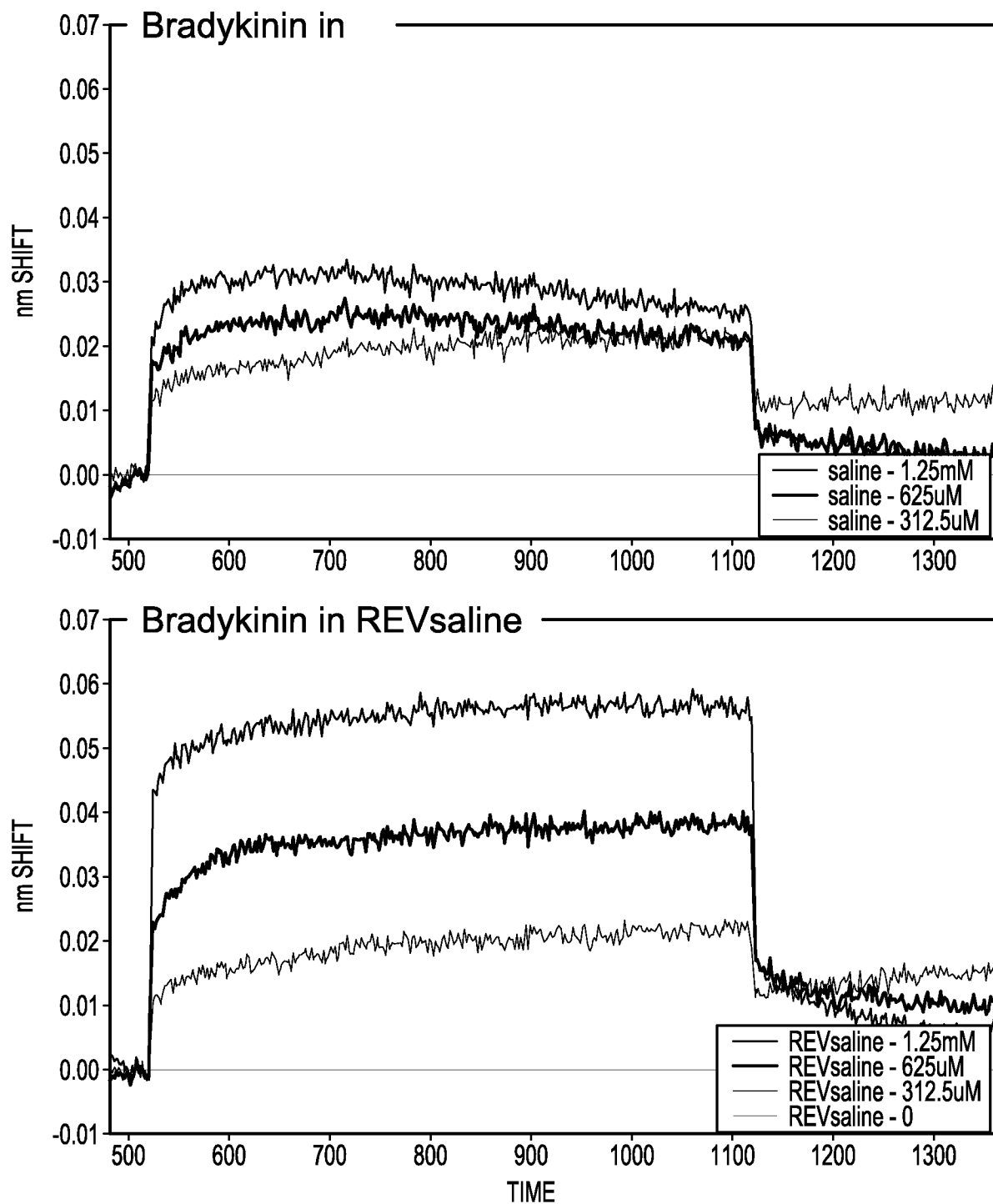


Fig. 70

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*Fig. 72*

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*Fig. 74*

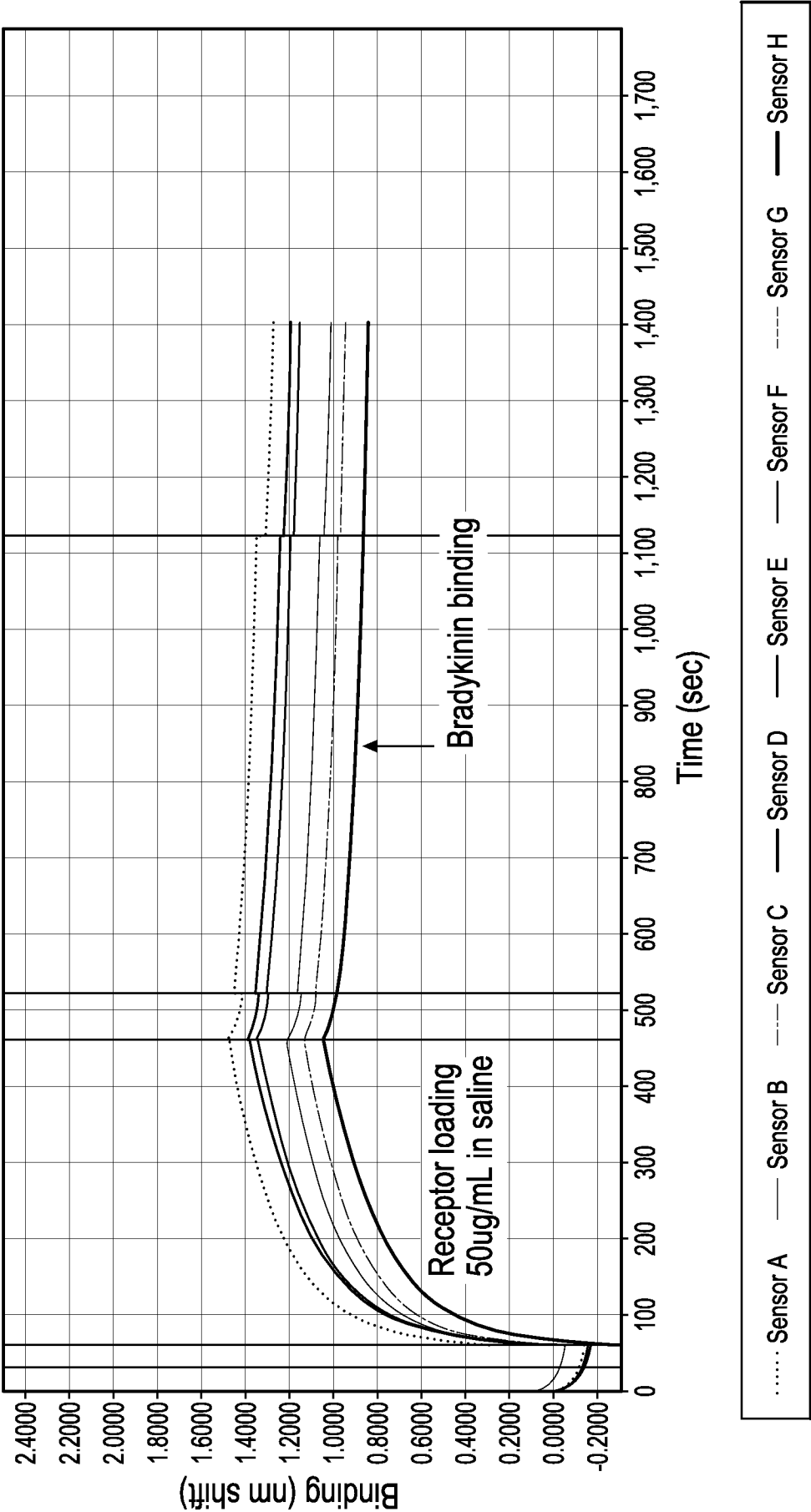


Fig. 75

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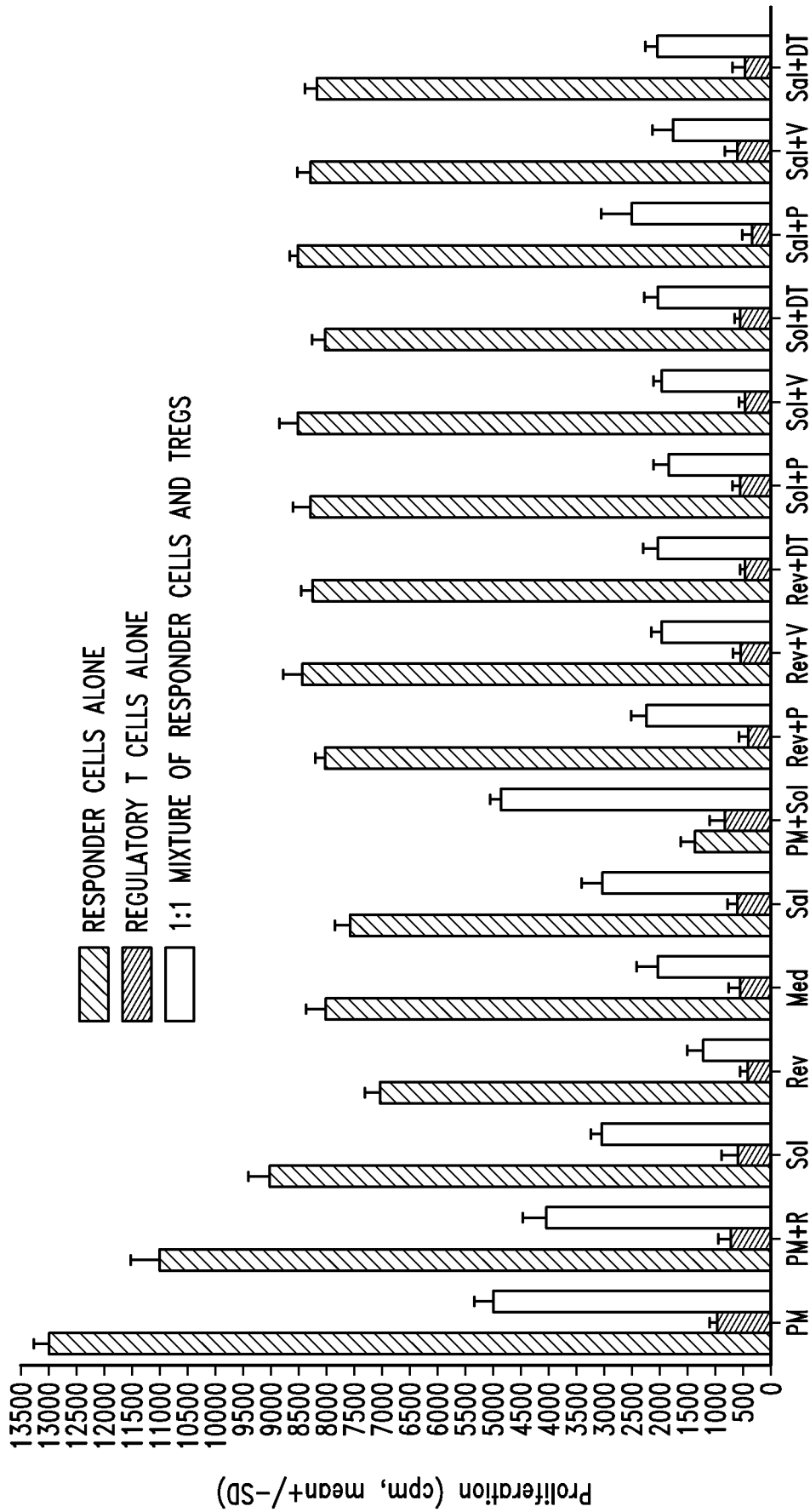
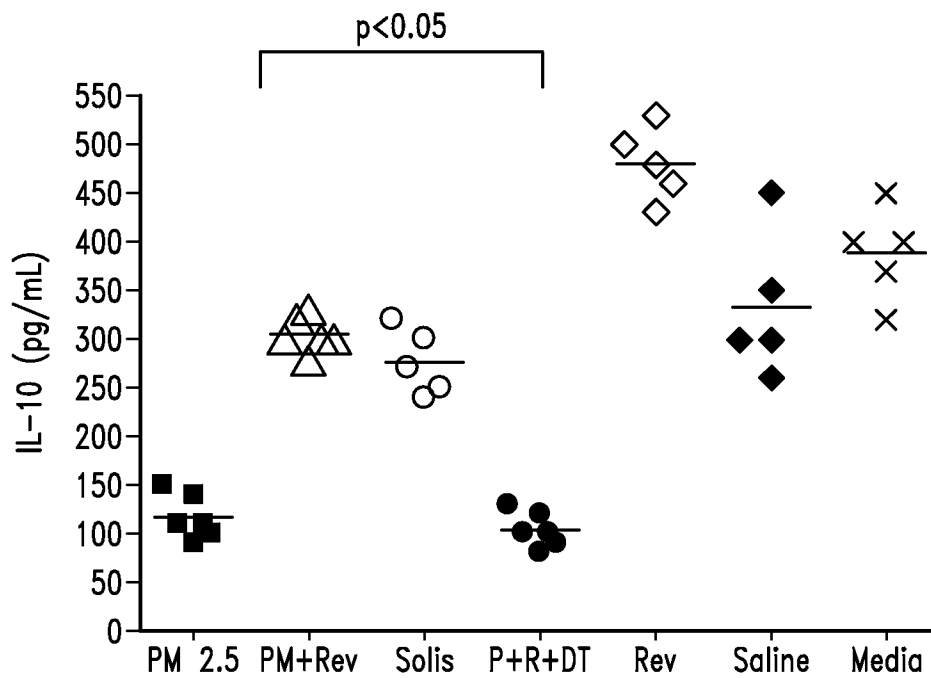
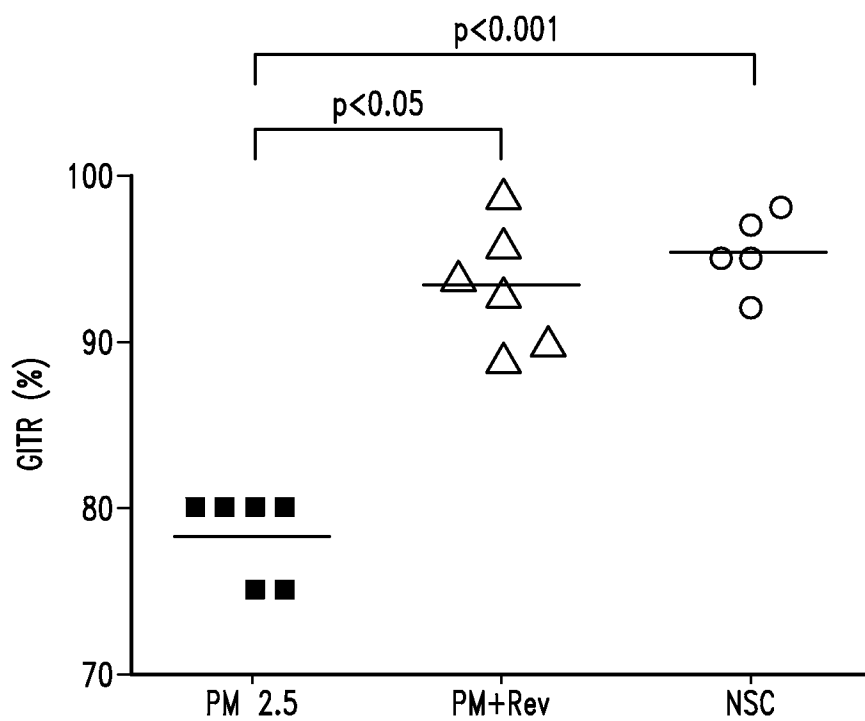
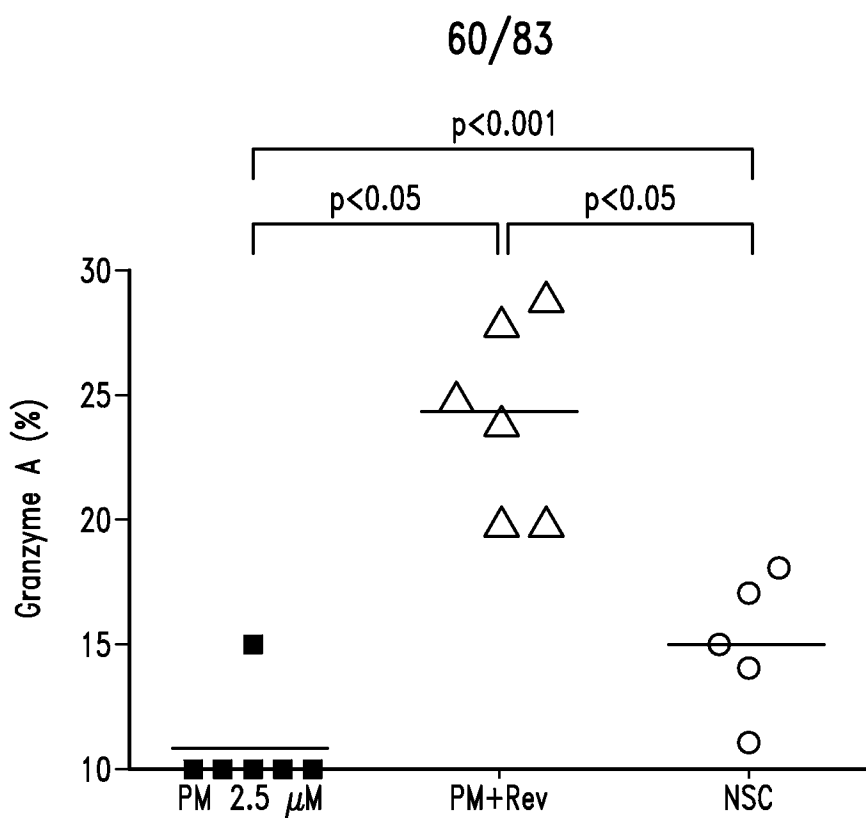
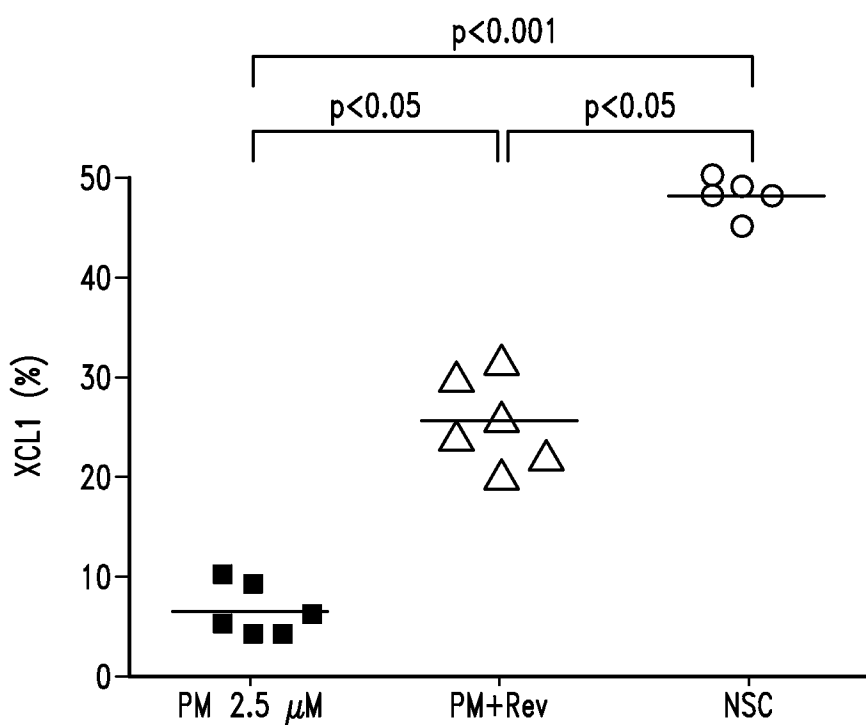
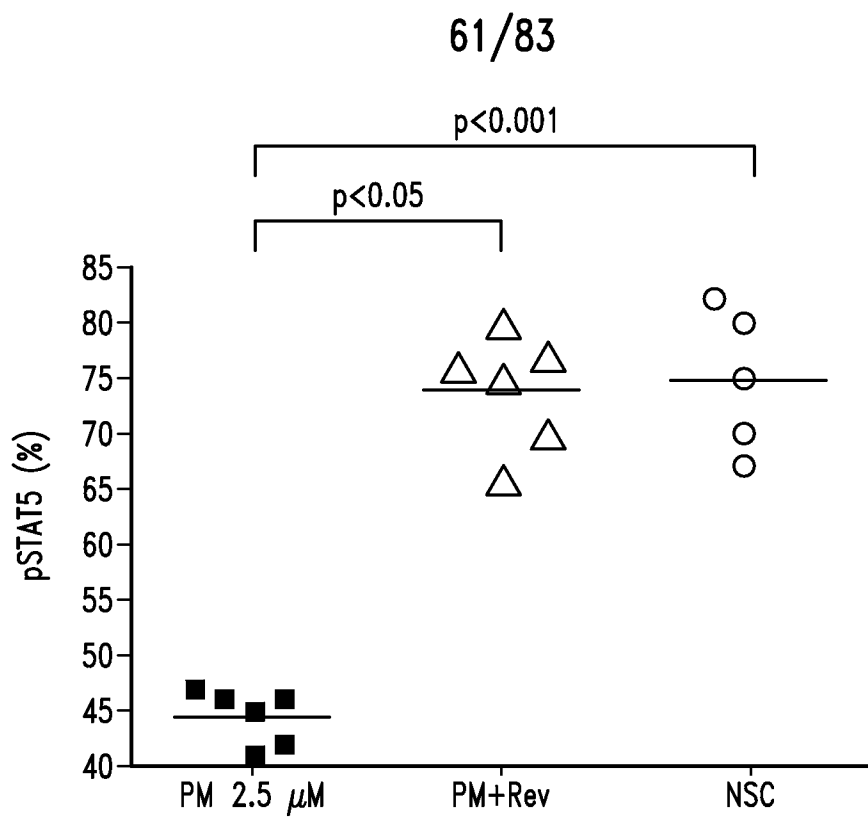
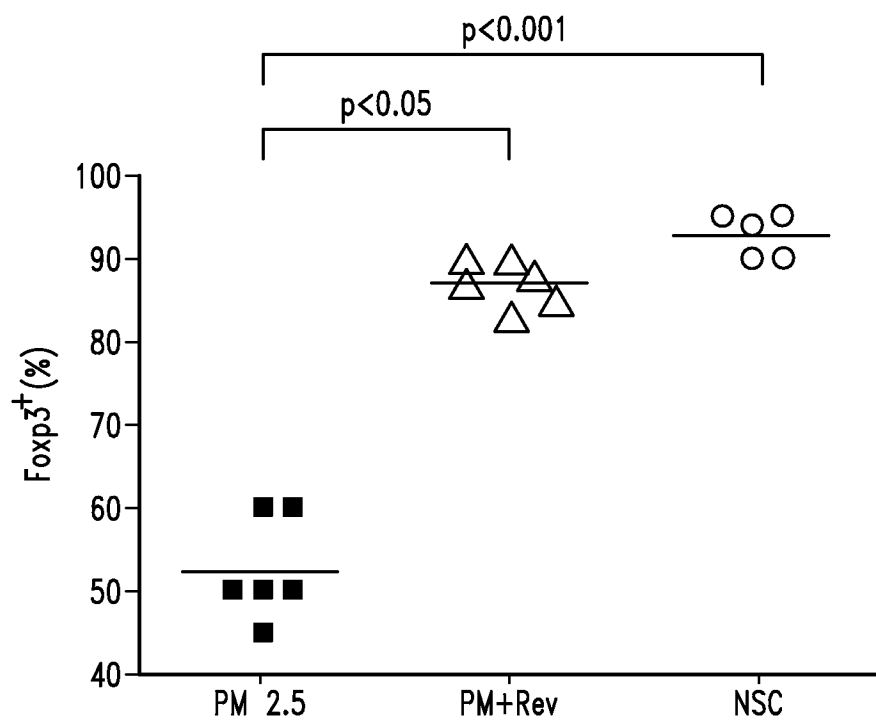


Fig. 76

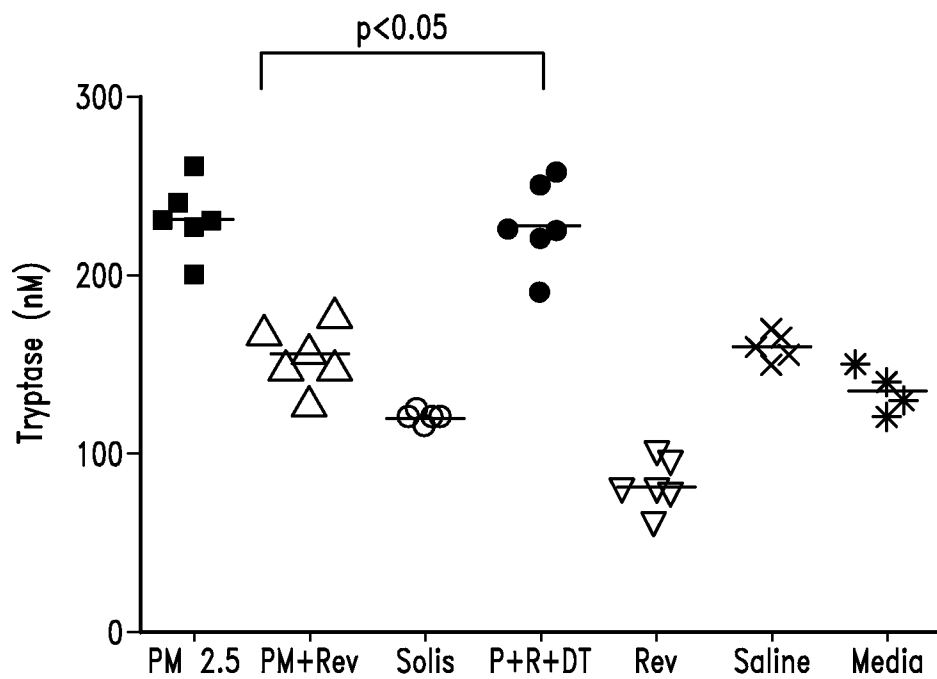
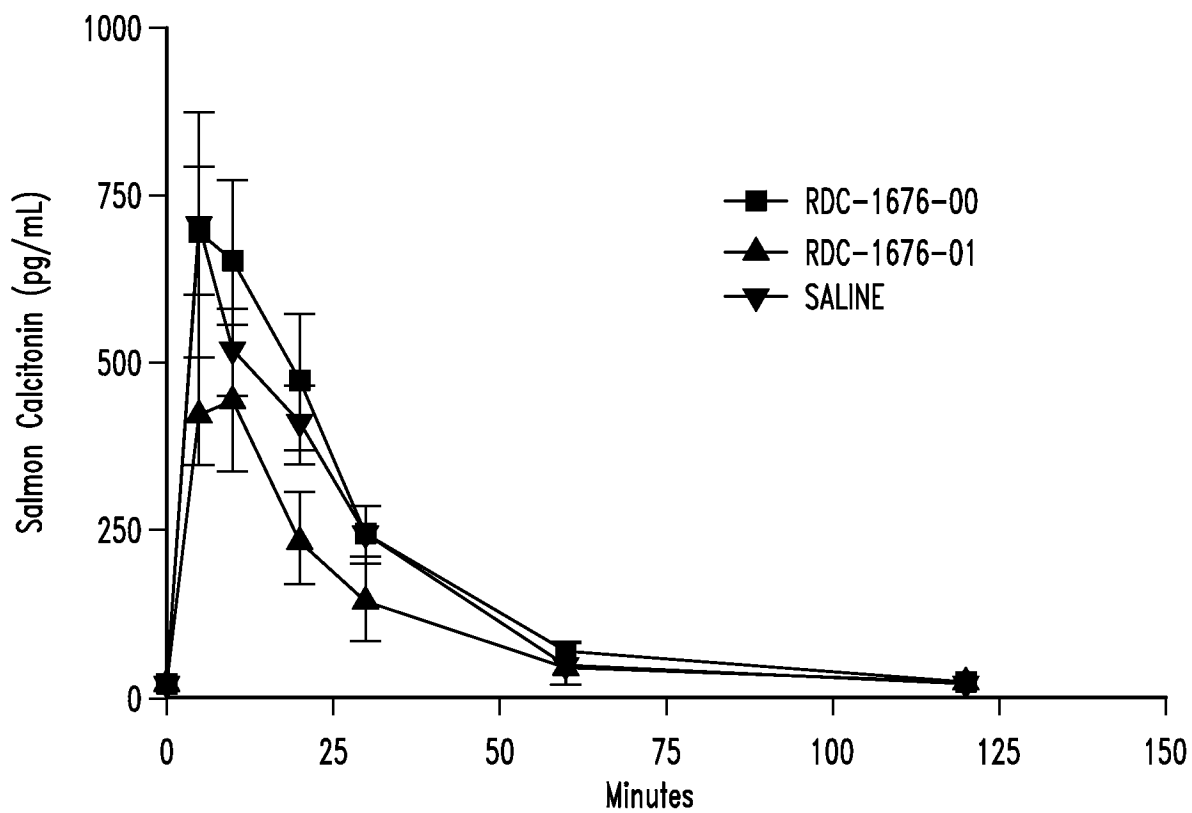
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*Fig. 77**Fig. 78*

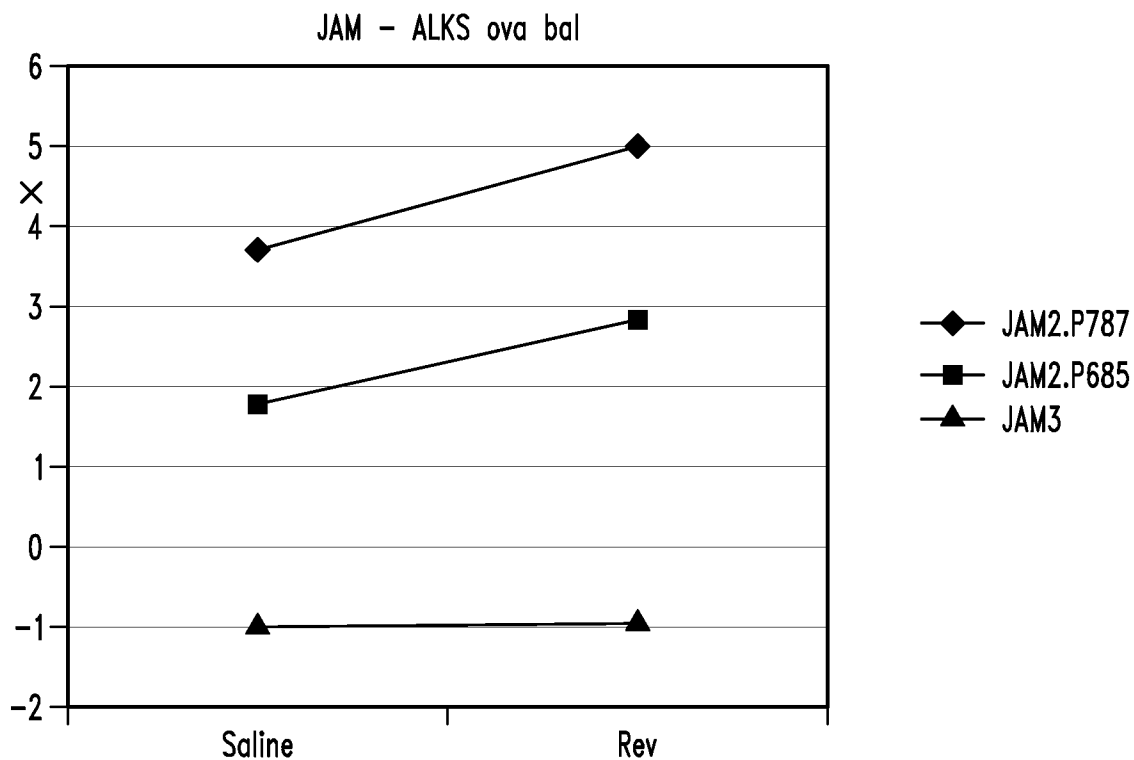
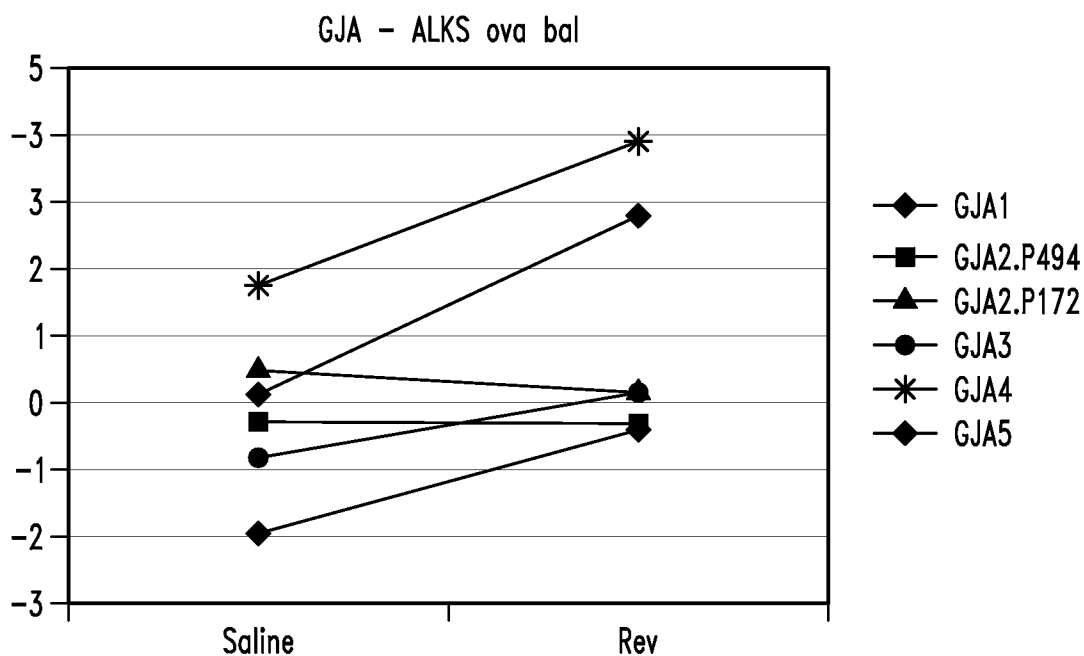
*Fig. 79**Fig. 80*

*Fig. 81**Fig. 82*

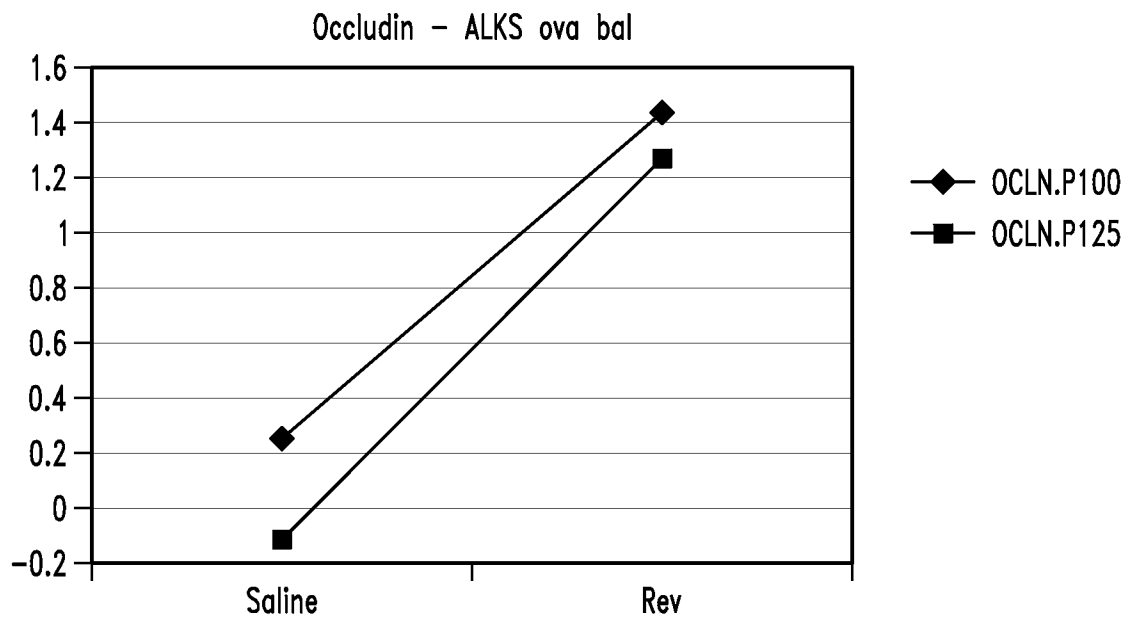
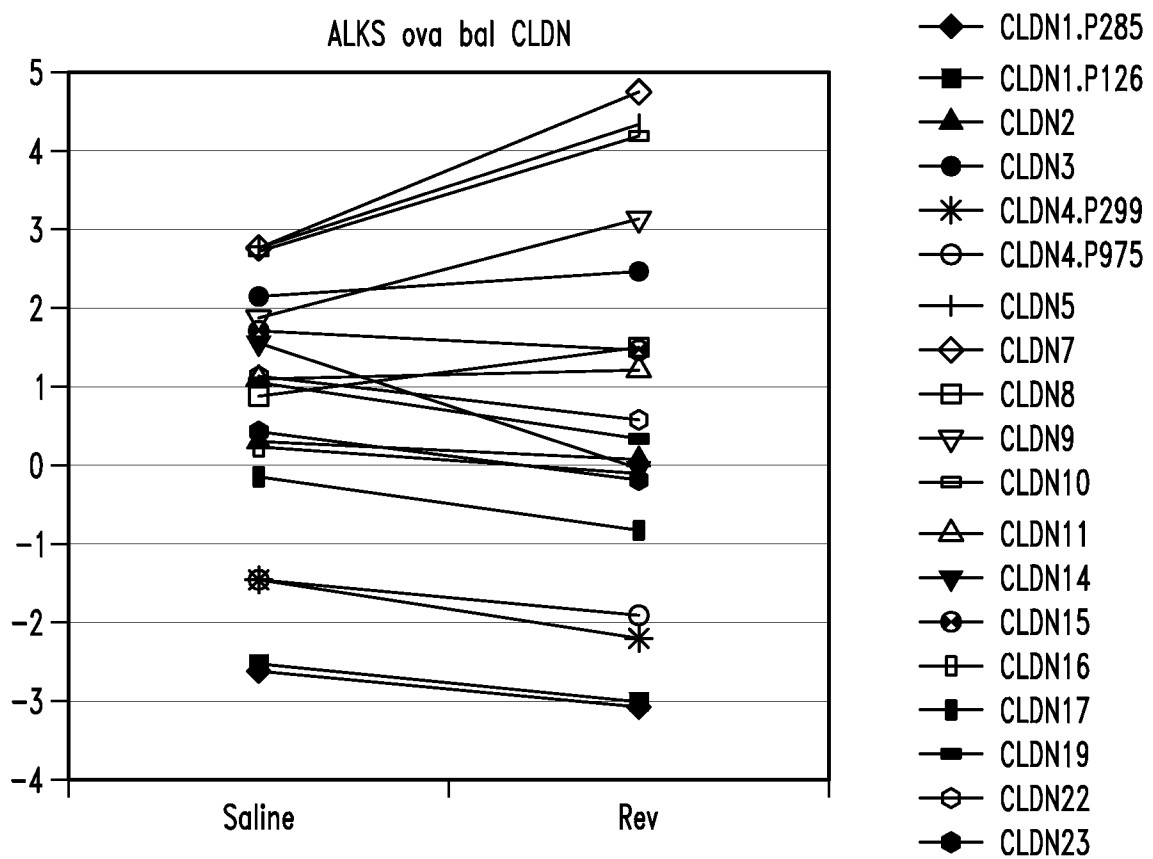
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*Fig. 83**Fig. 84*

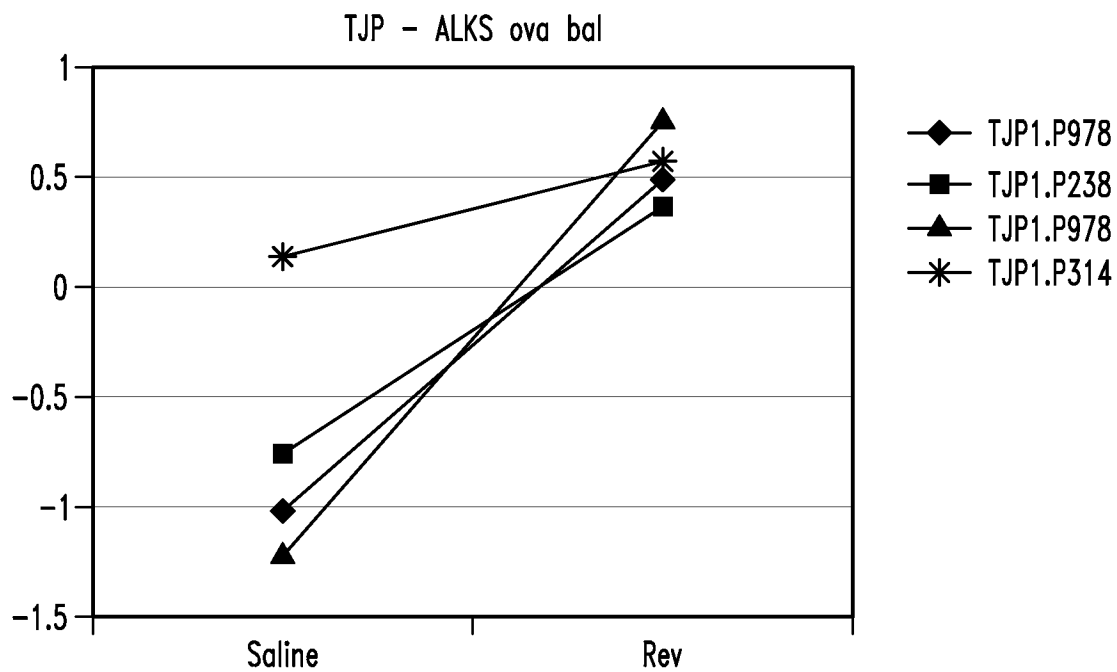
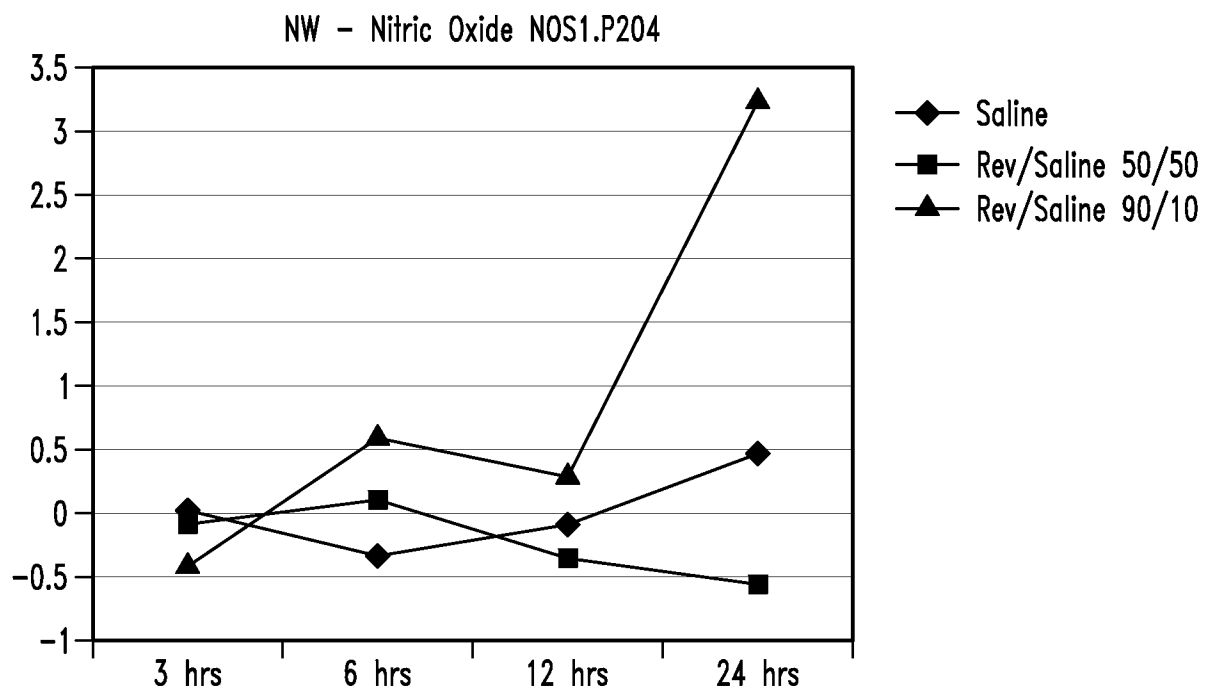
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*Fig. 85**Fig. 86*

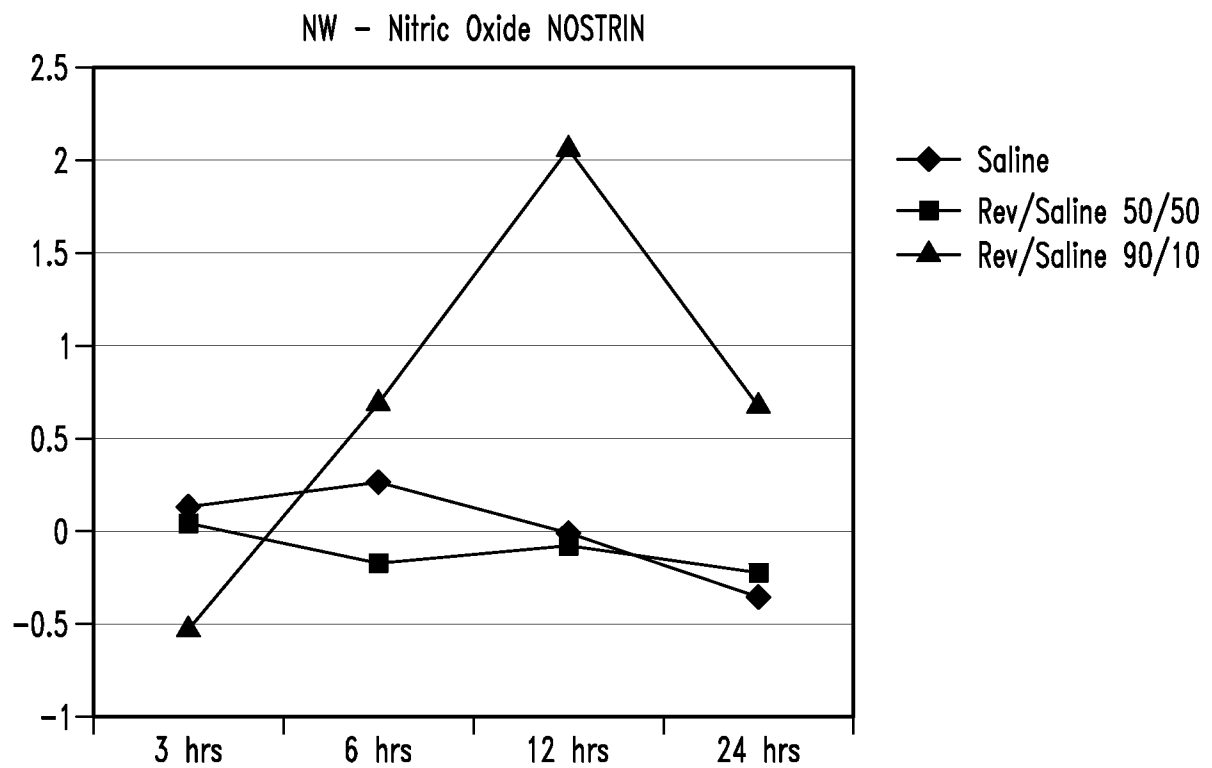
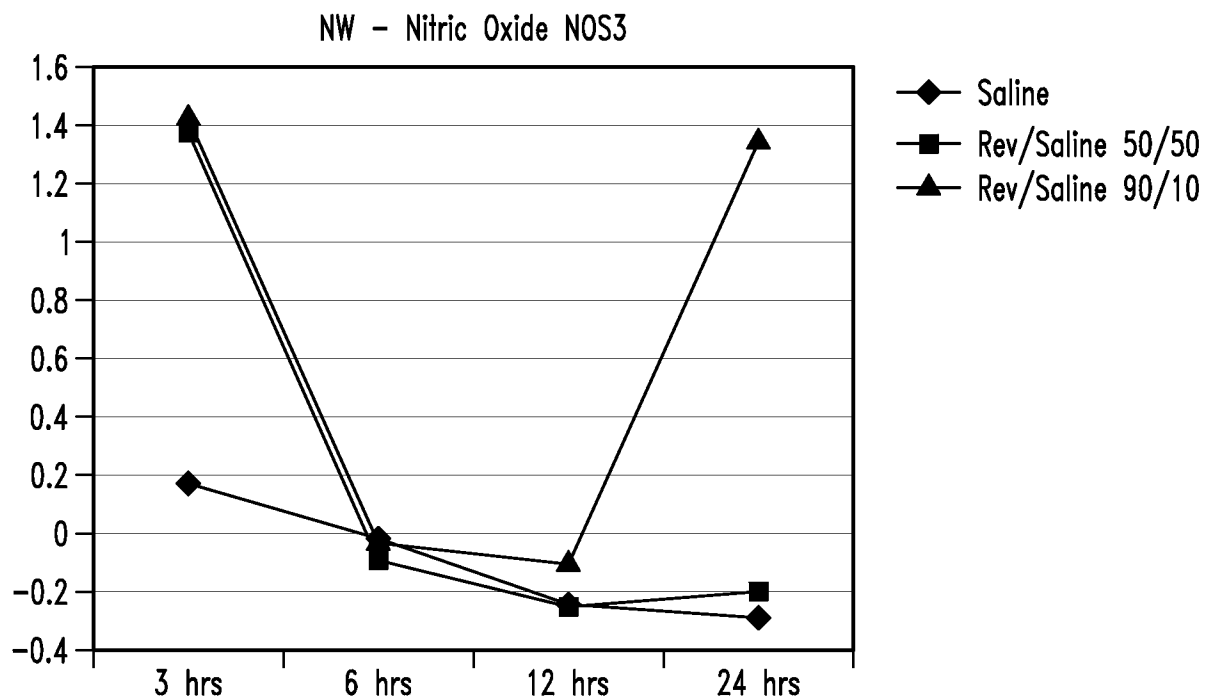
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*Fig. 87**Fig. 88*

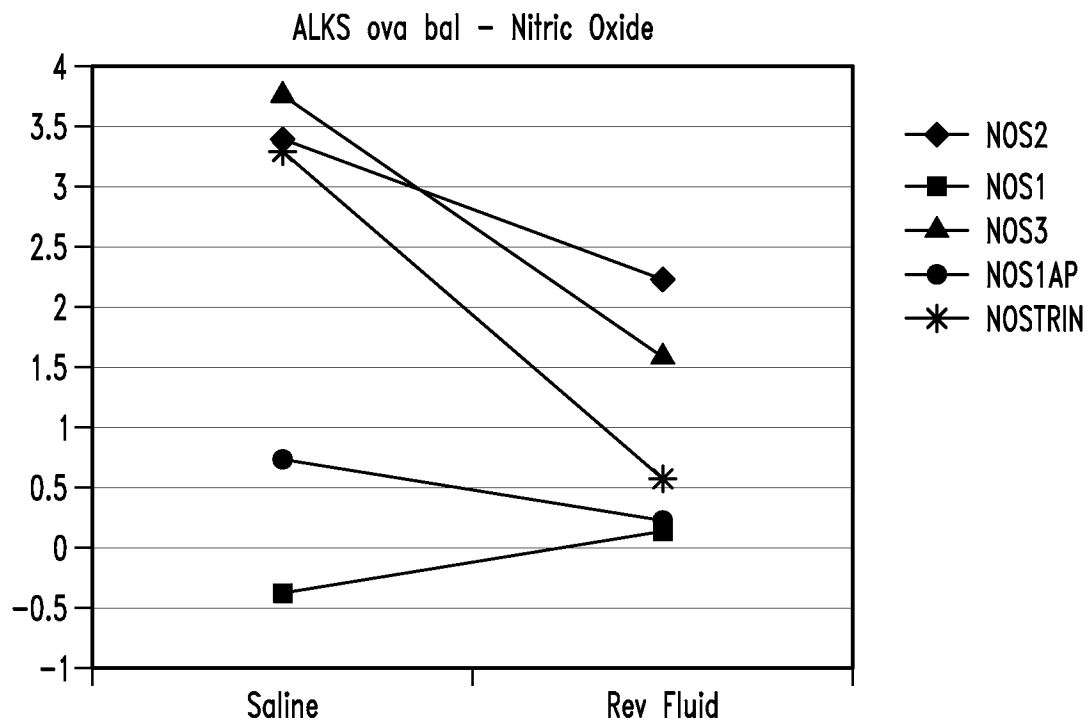
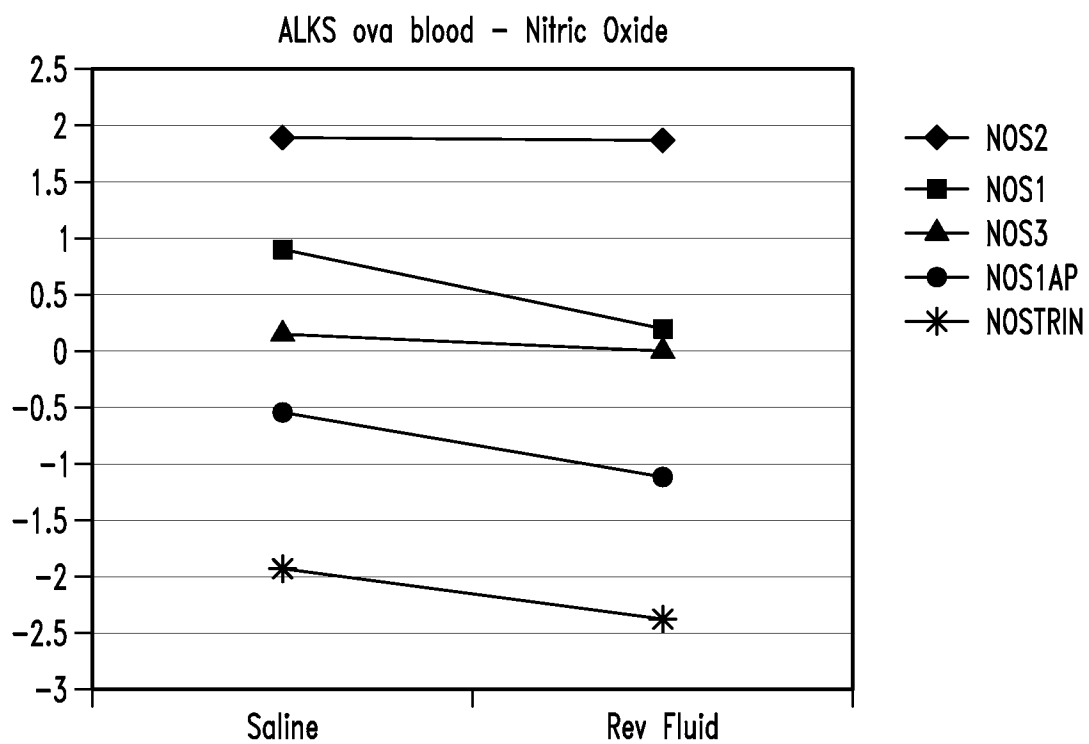
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*Fig. 89**Fig. 90*

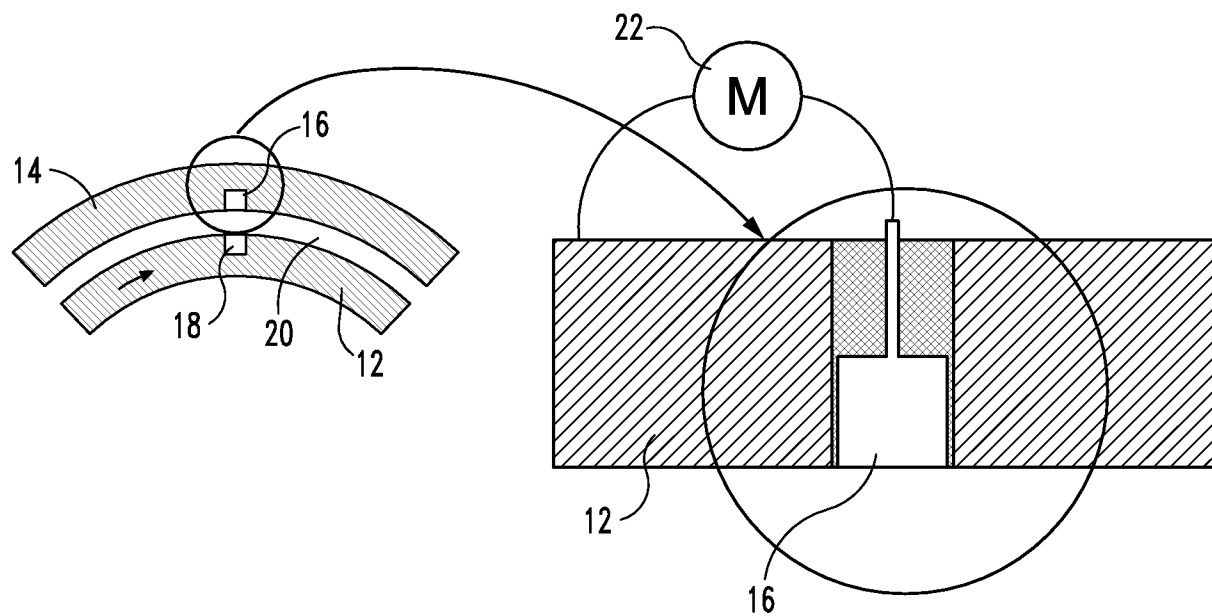
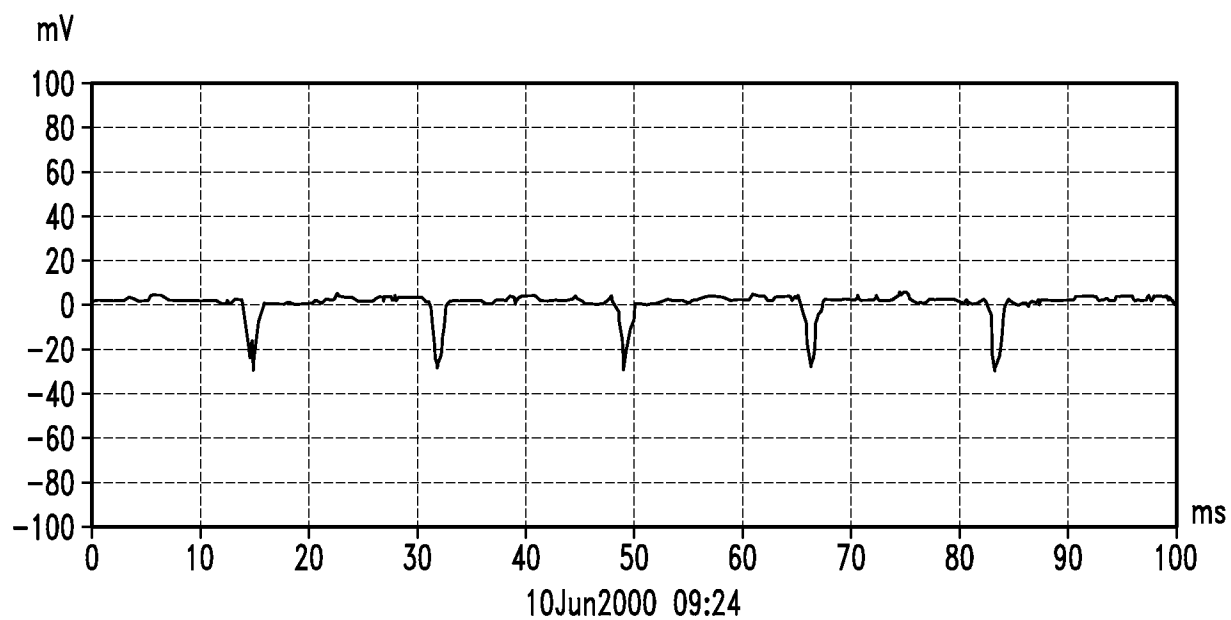
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*Fig. 91**Fig. 92*

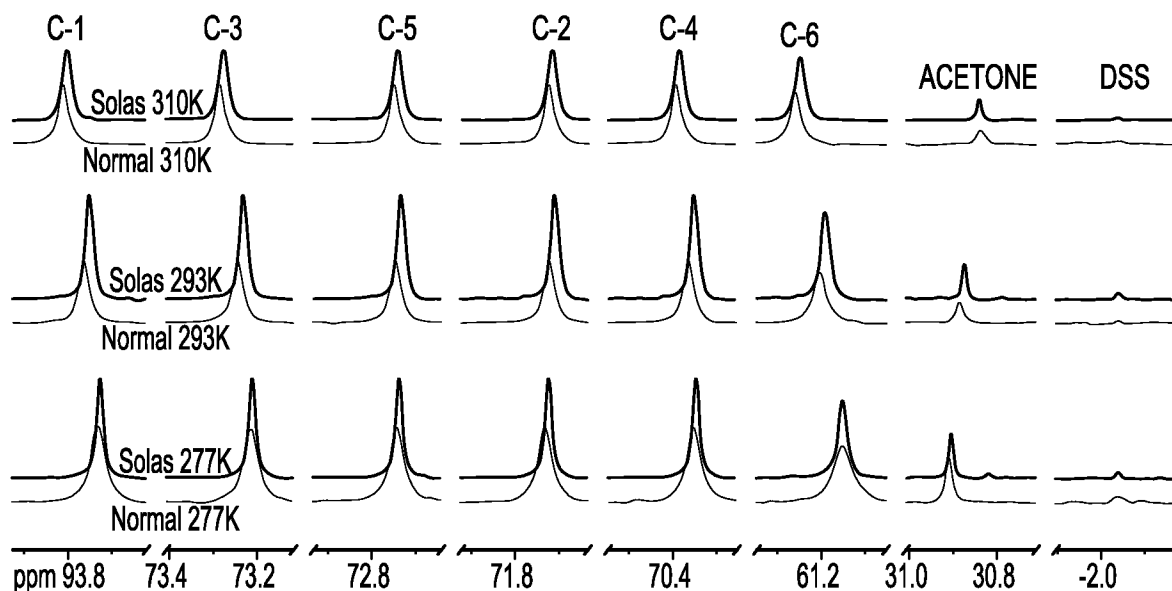
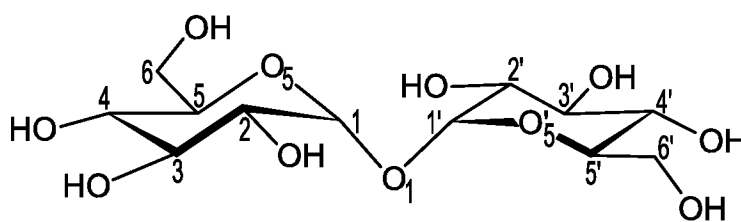
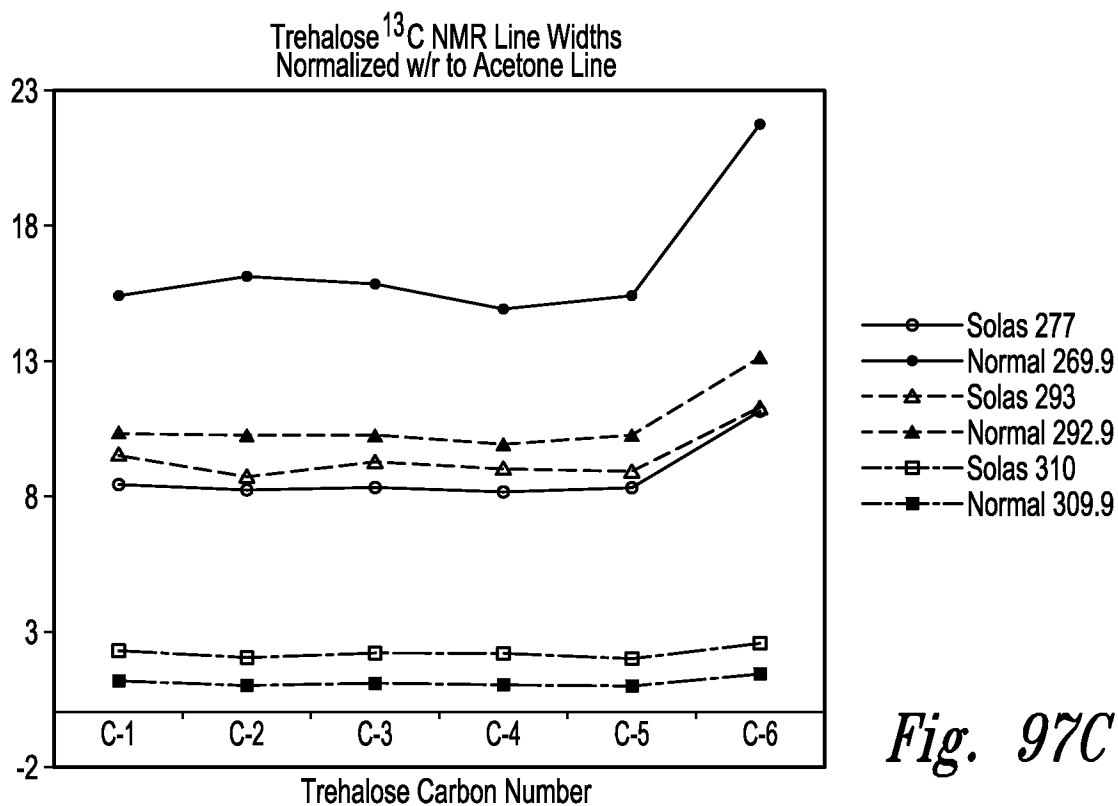
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*Fig. 93**Fig. 94*

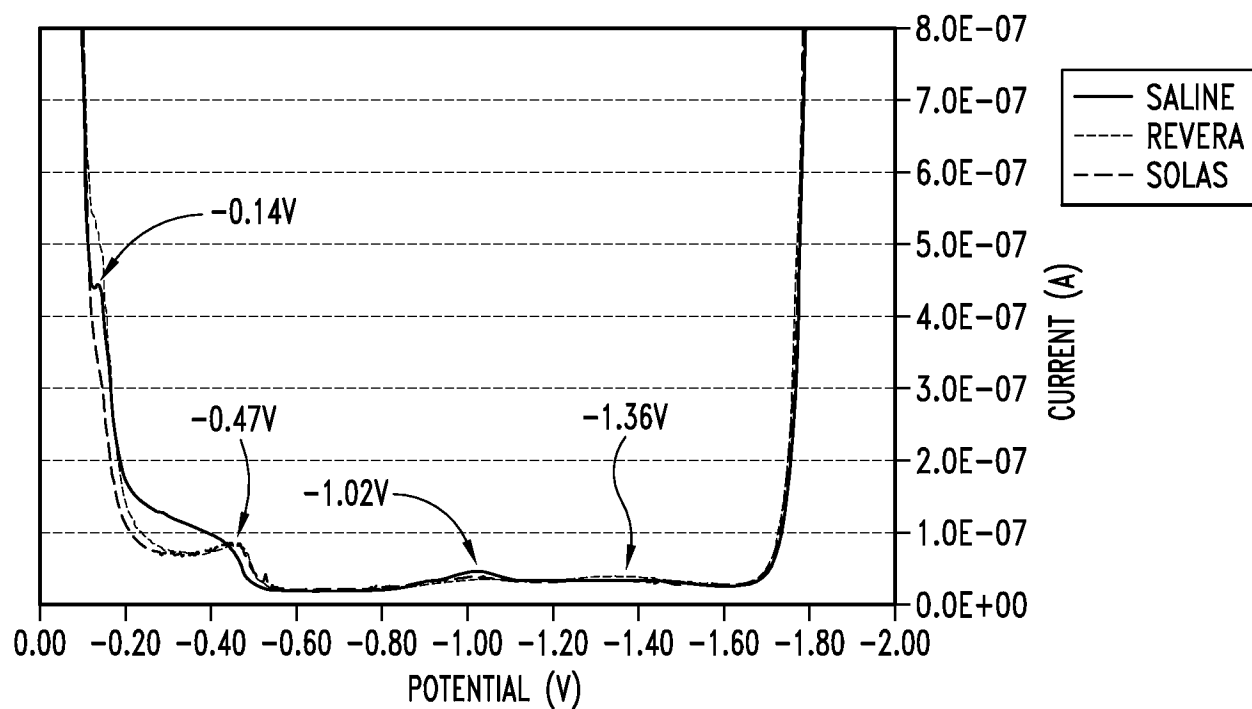
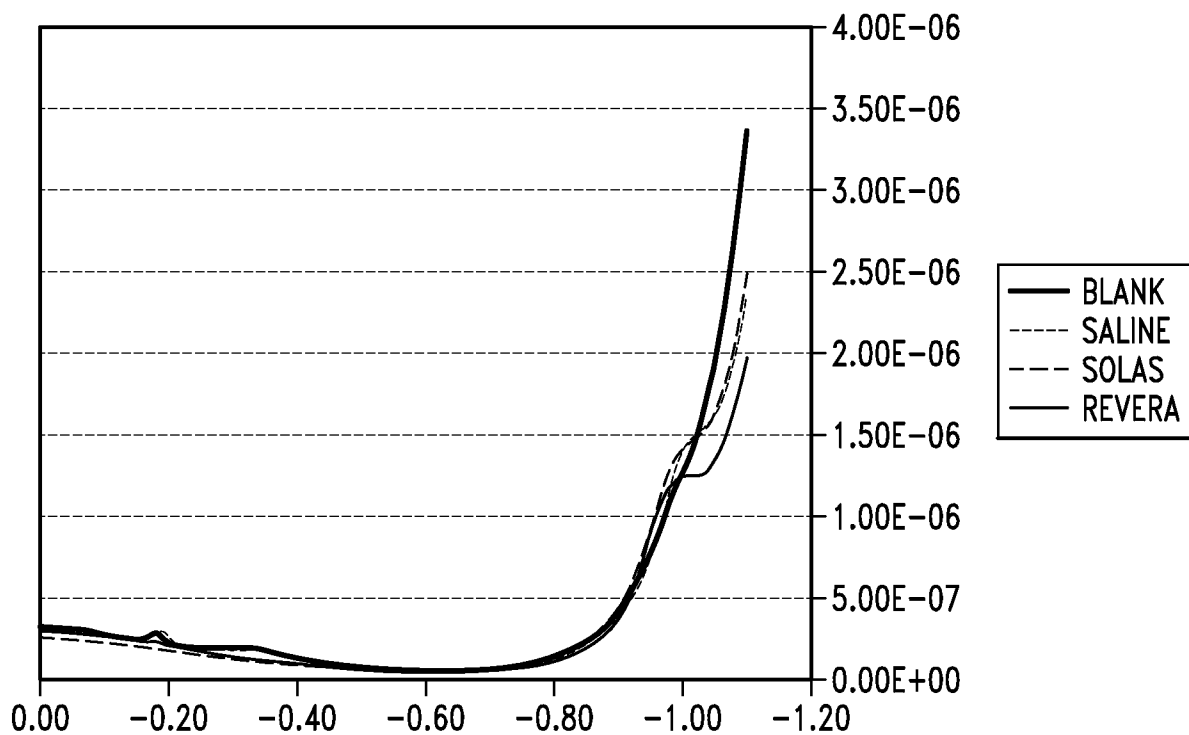
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*Fig. 95**Fig. 96*

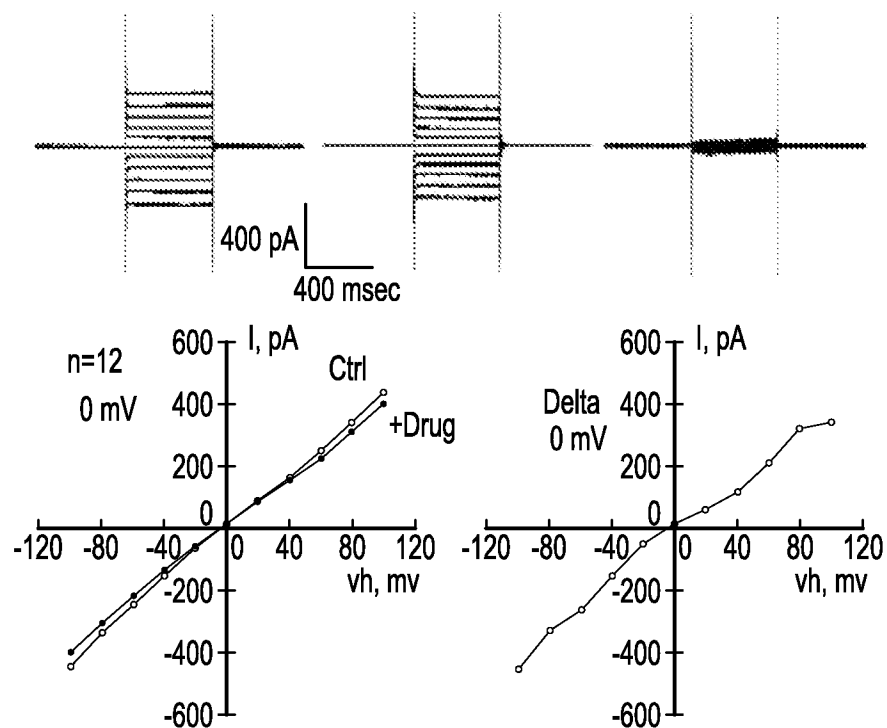
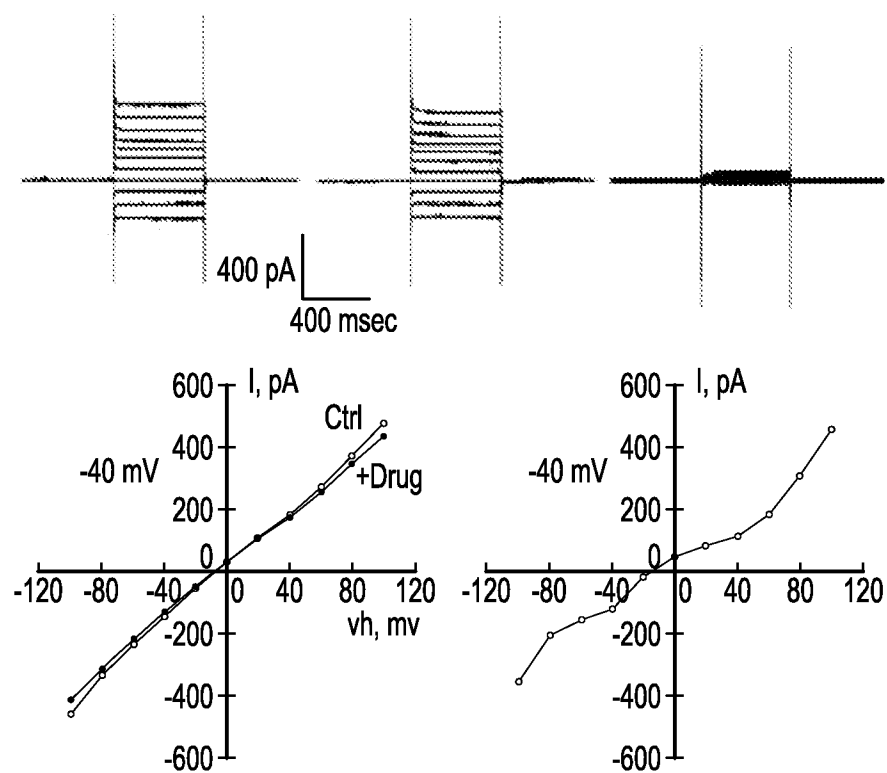
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*Fig. 97A**Fig. 97B**Fig. 97C*

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*Fig. 98**Fig. 99*

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*Fig. 100**Fig. 101*

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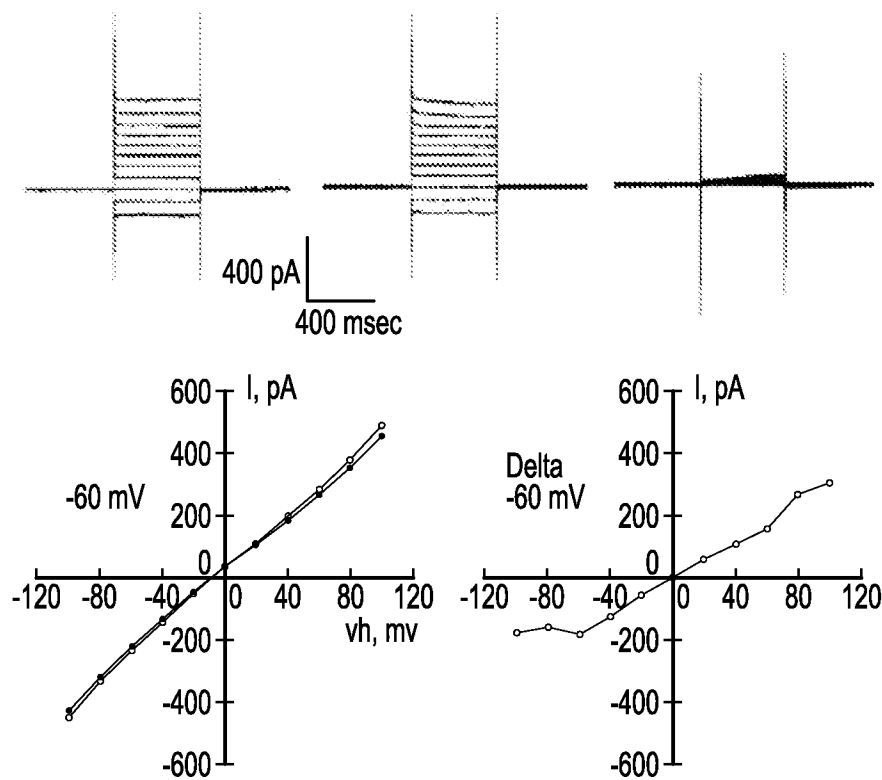


Fig. 102

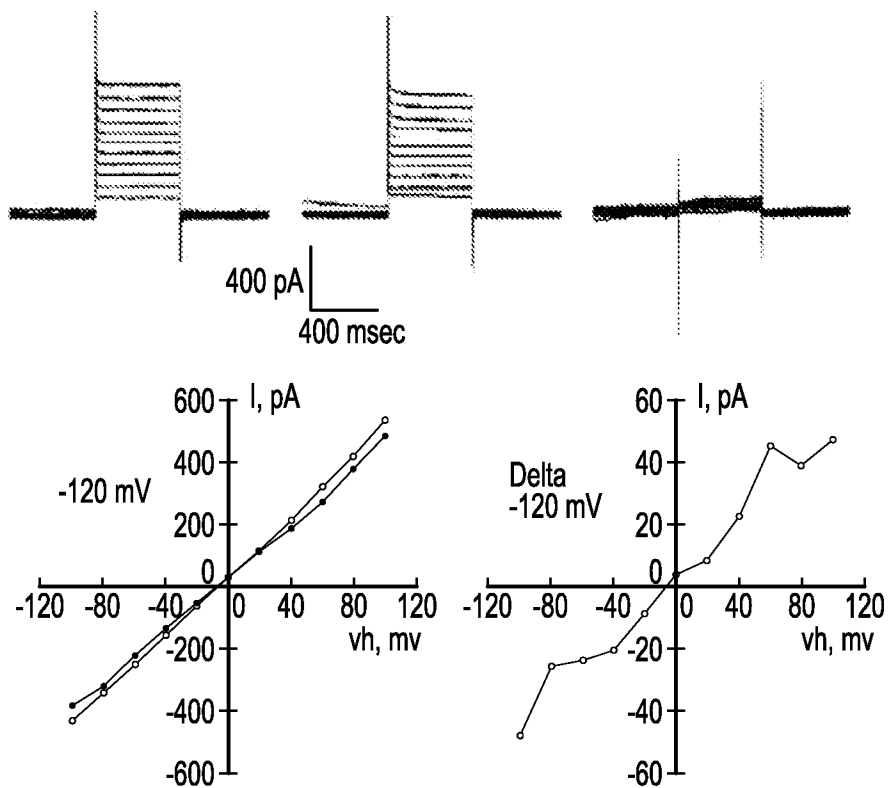
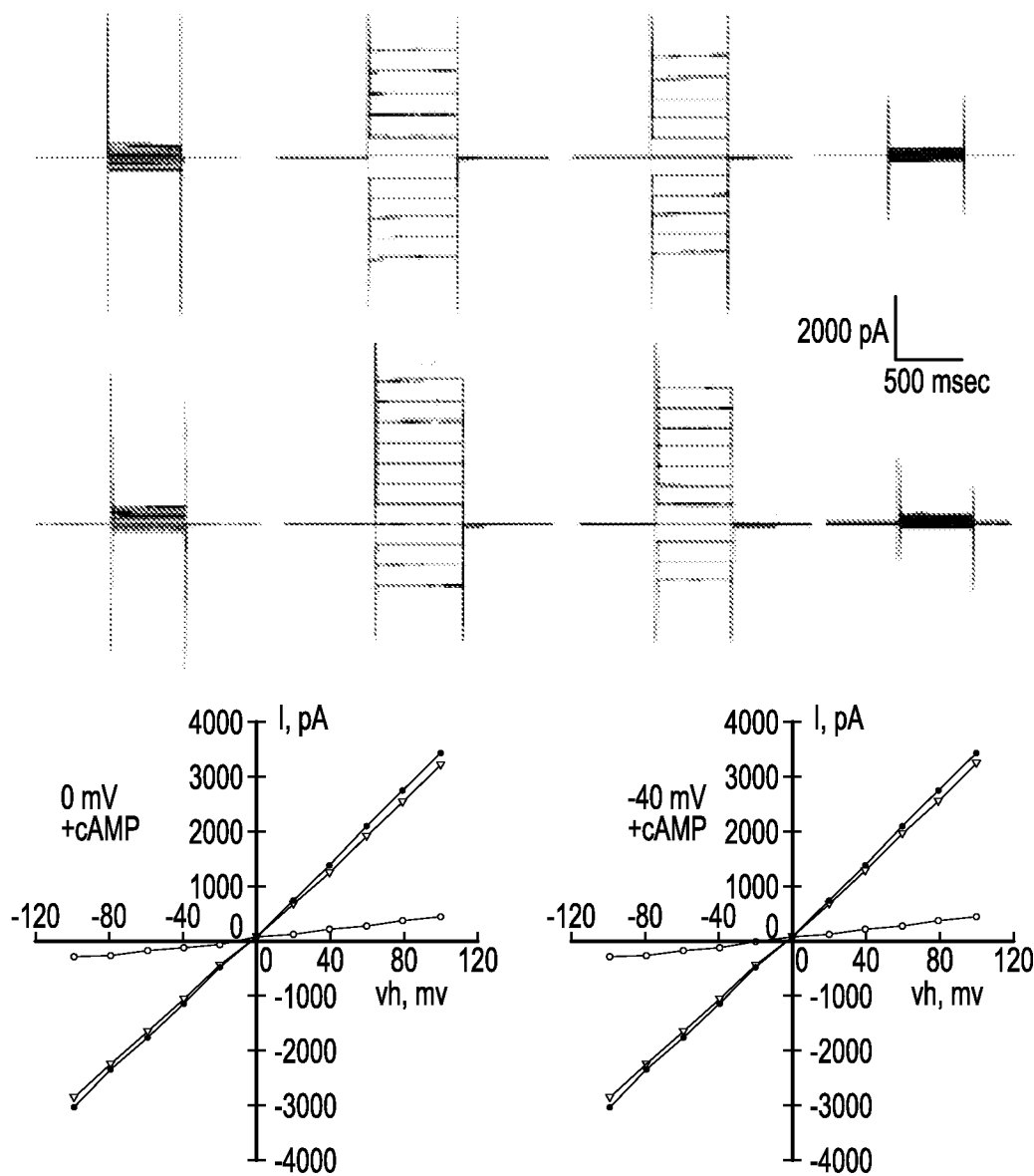
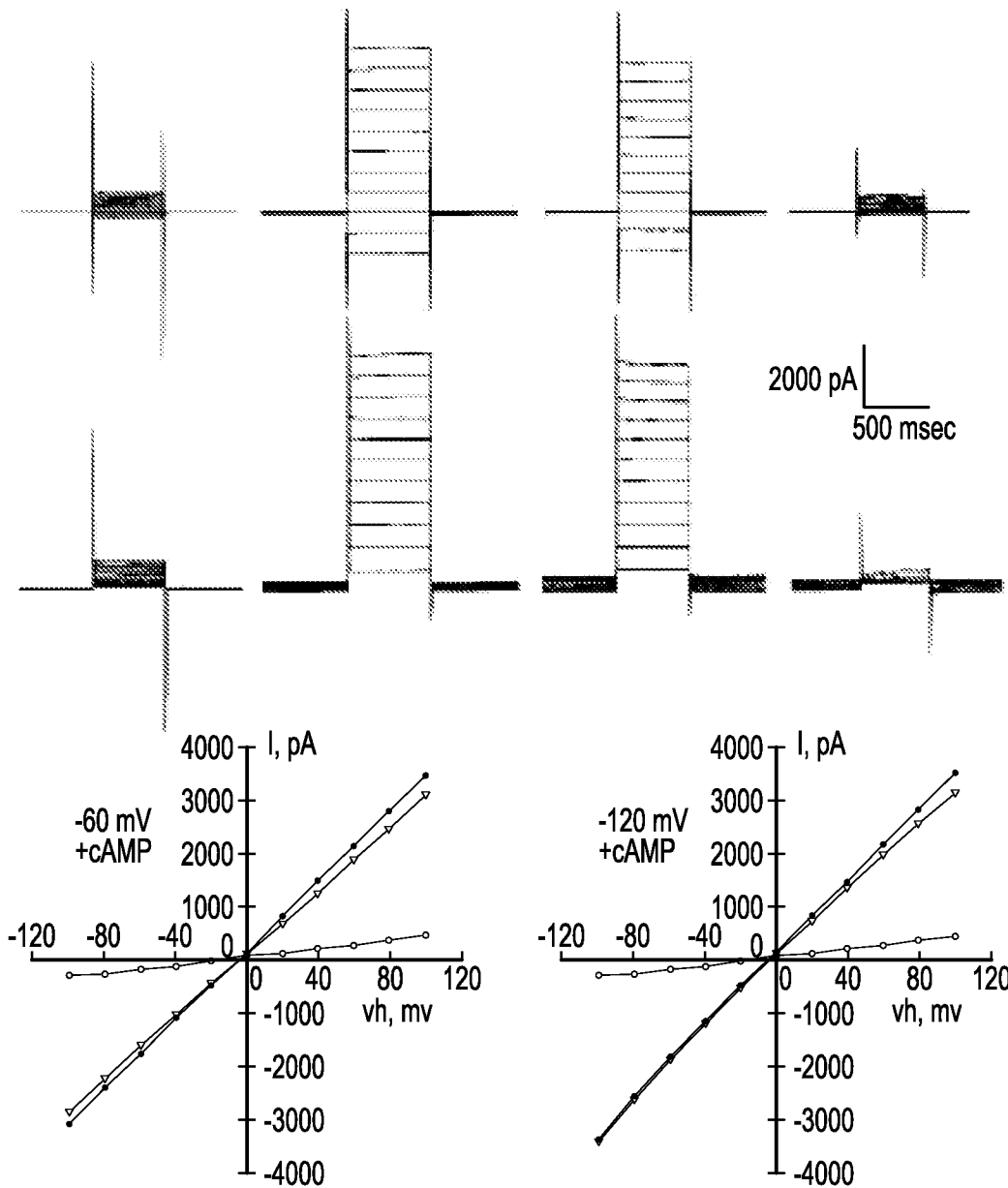


Fig. 103

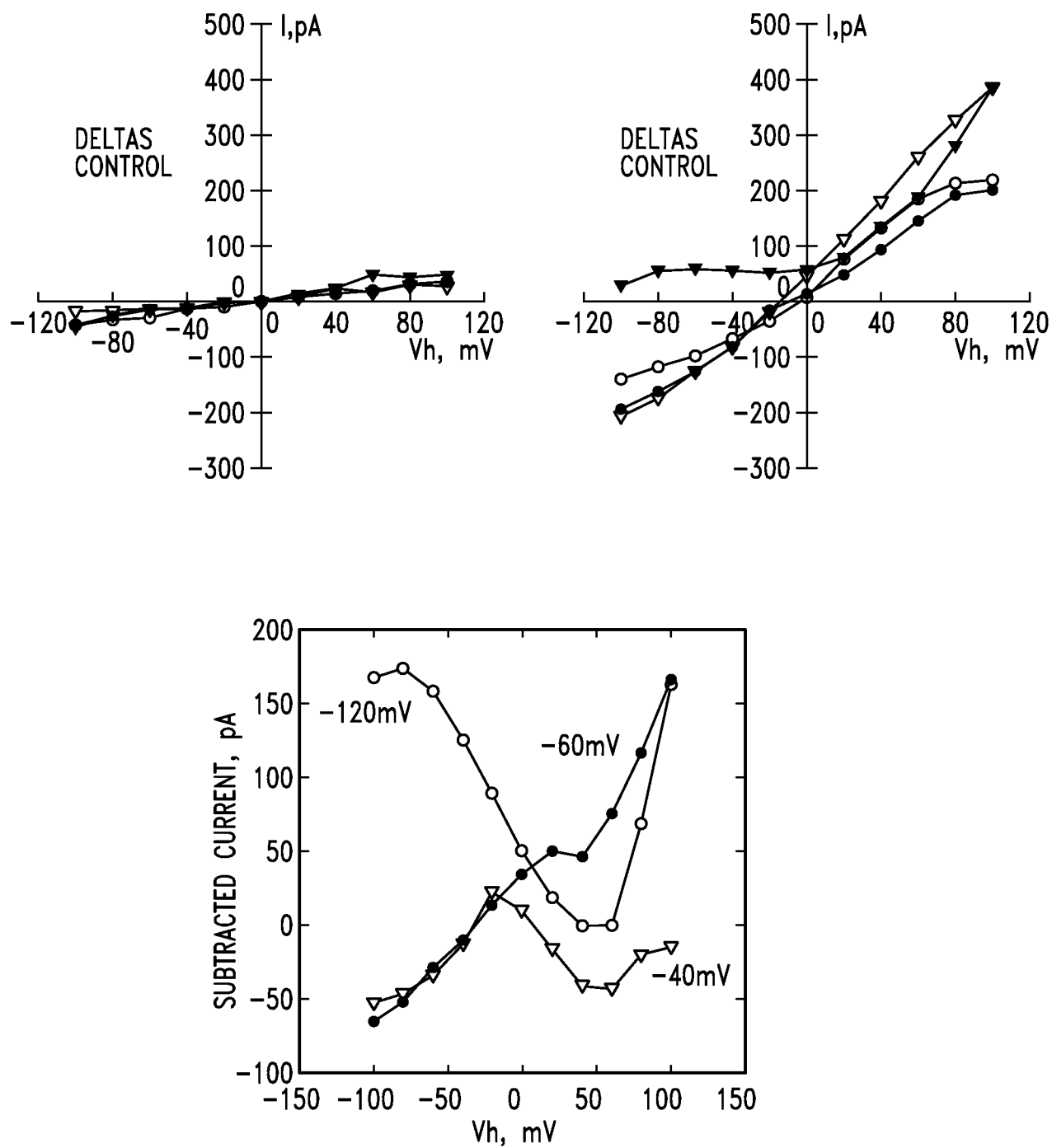
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*Fig. 104*

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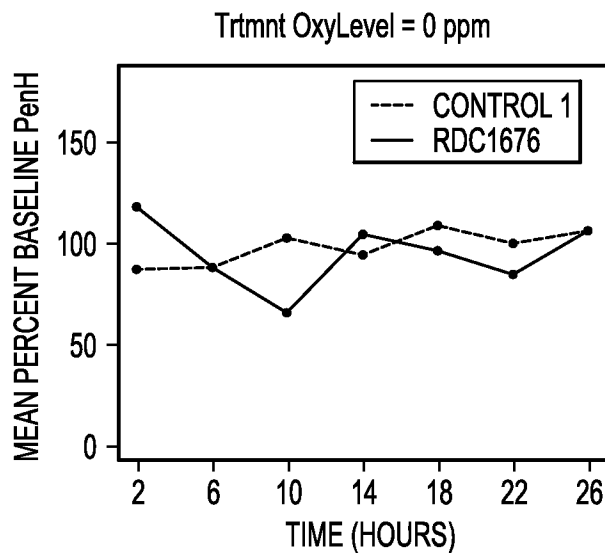
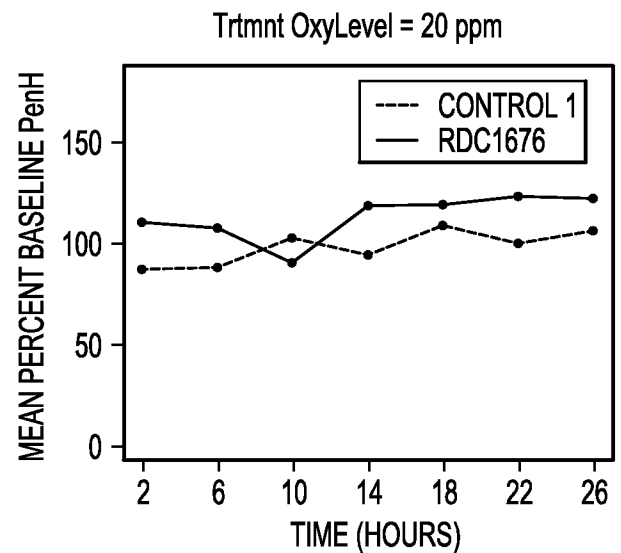
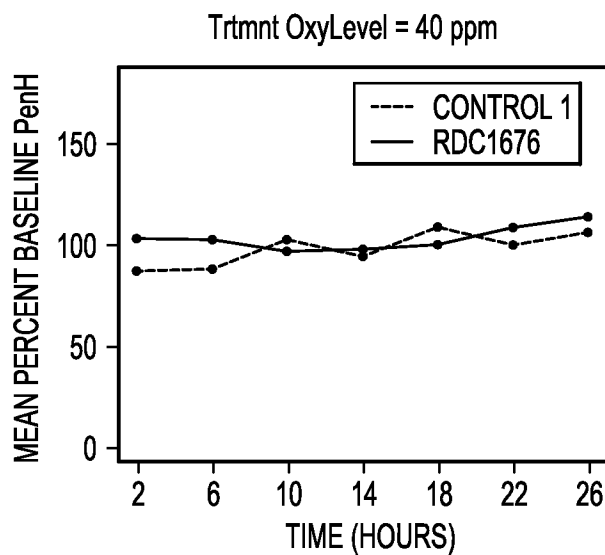
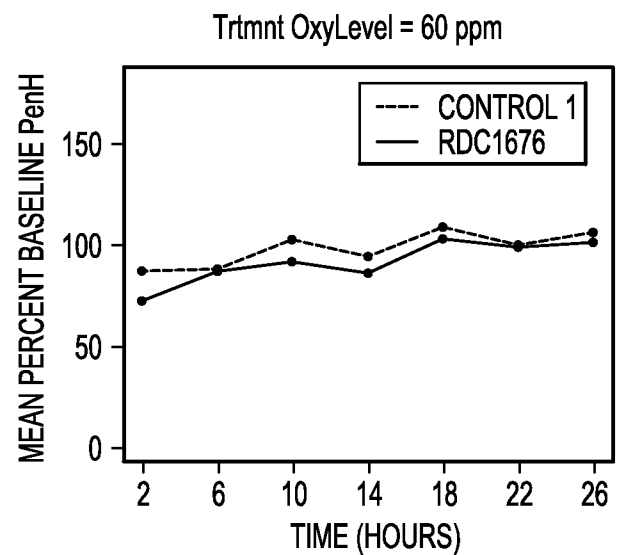
*Fig. 105*

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*Fig. 106*

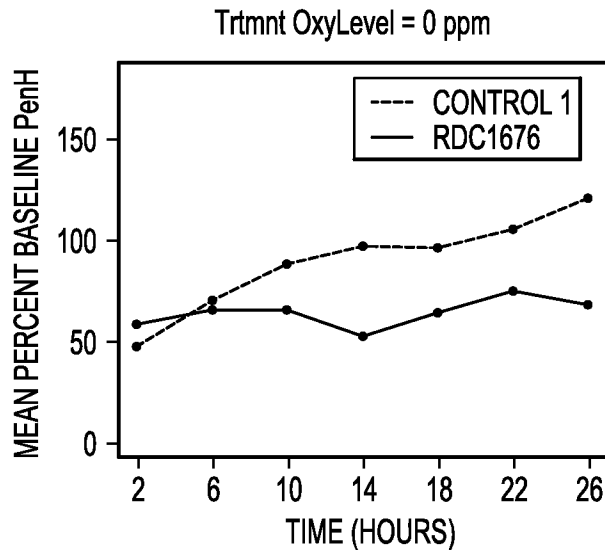
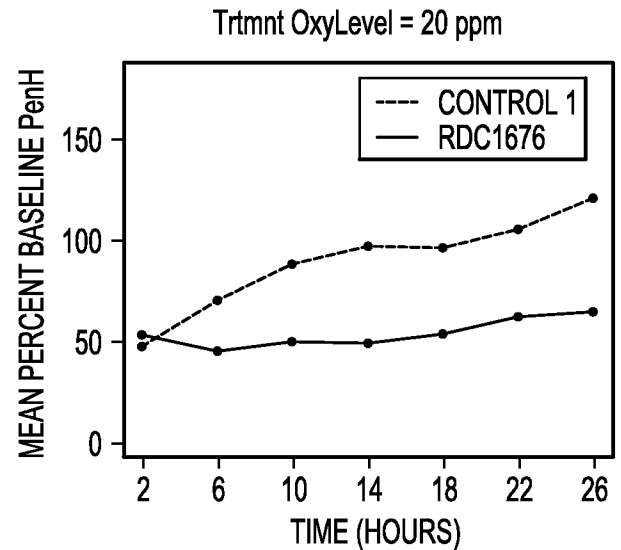
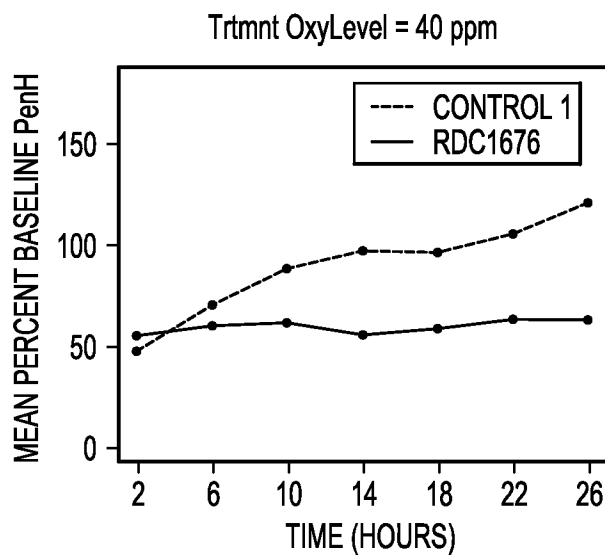
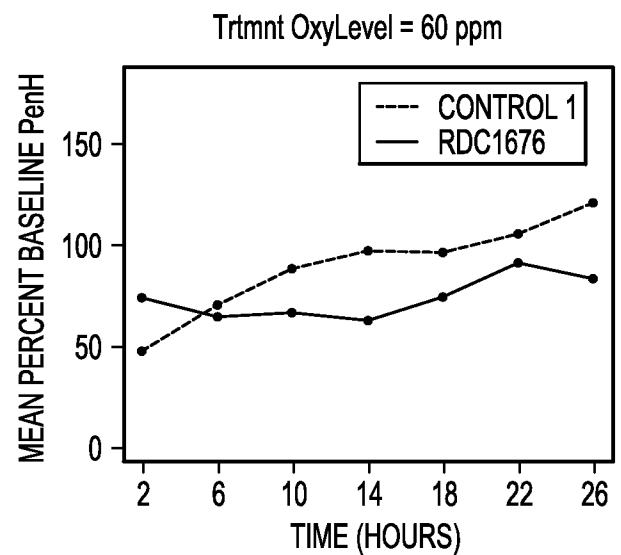
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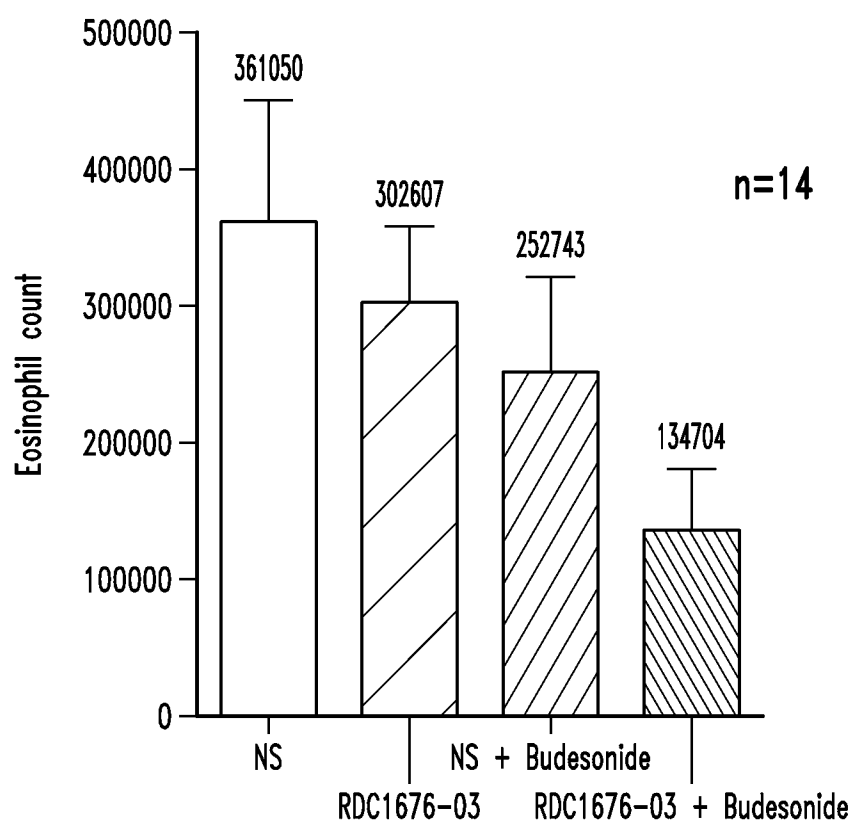
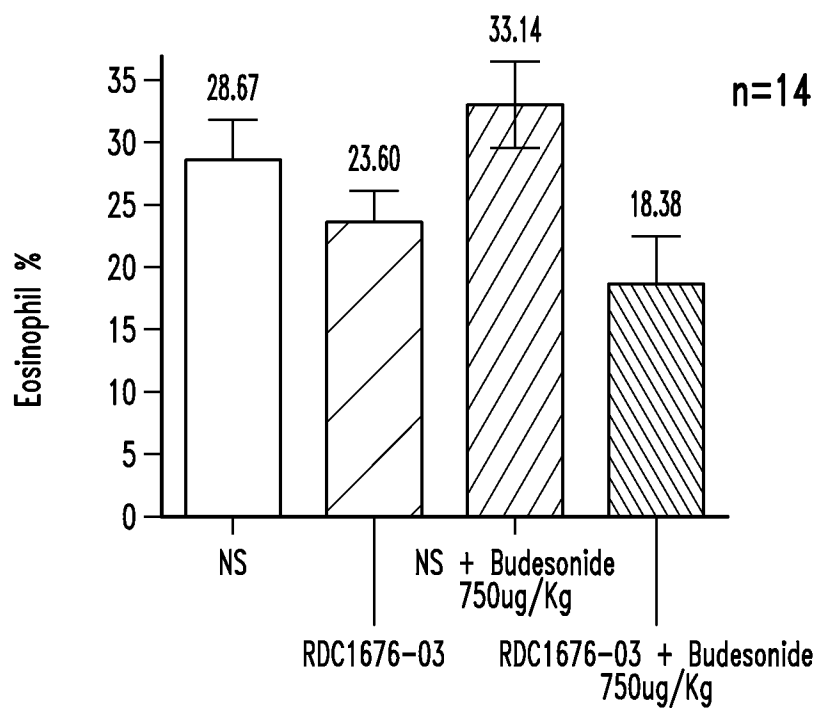
*Fig. 107A**Fig. 107B**Fig. 107C**Fig. 107D*

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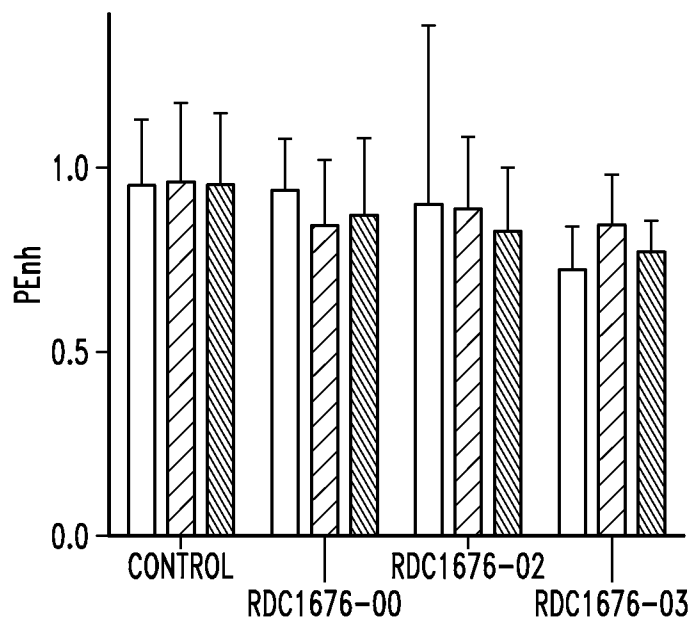
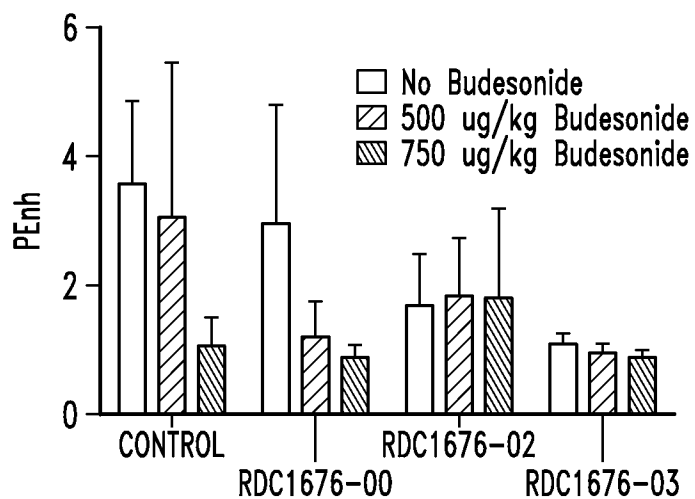
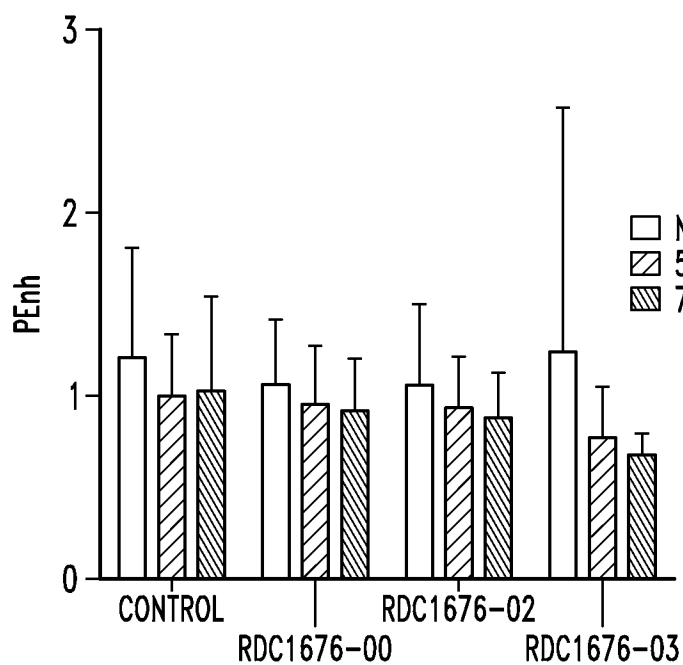
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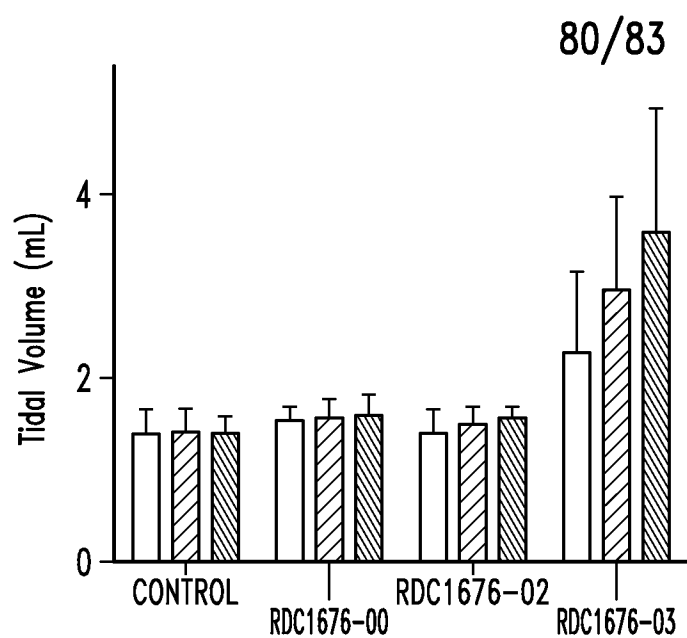
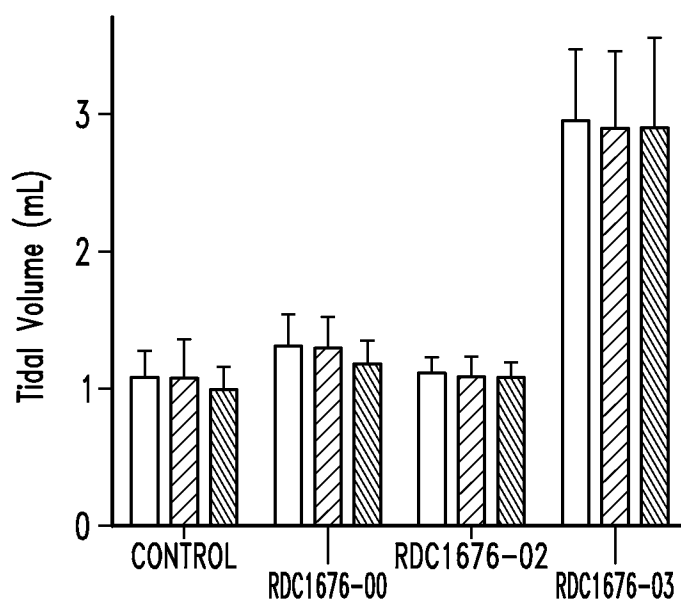
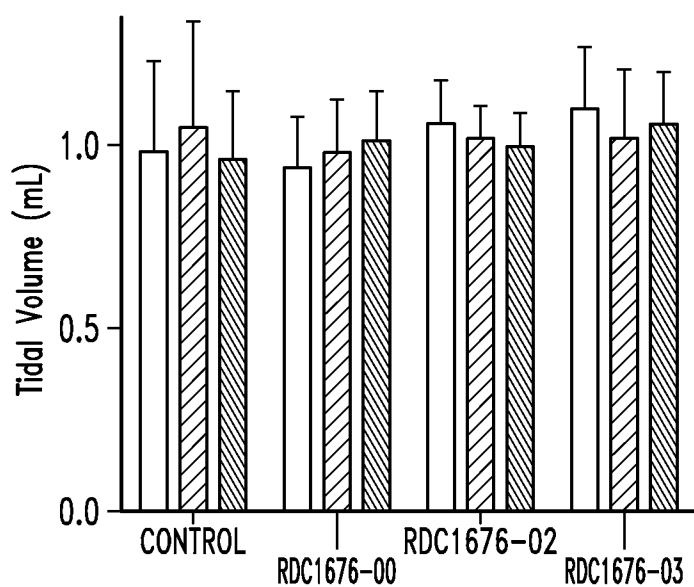
*Fig. 108A**Fig. 108B**Fig. 108C**Fig. 108D*

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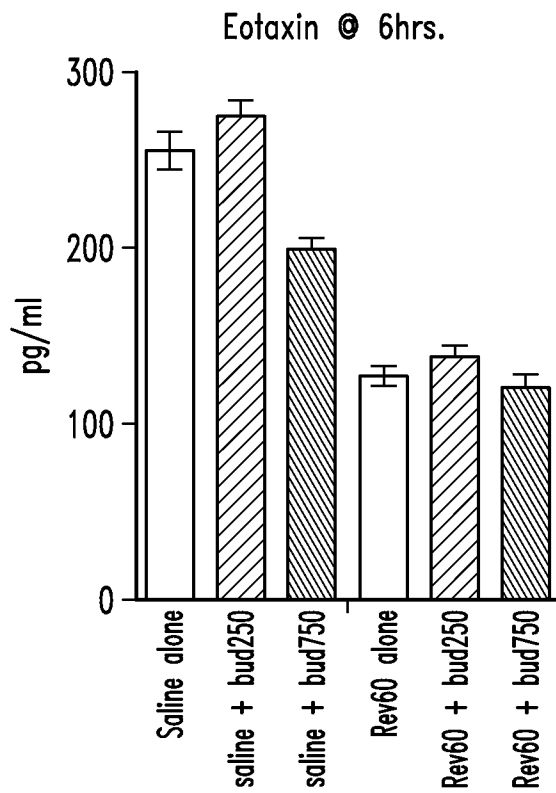
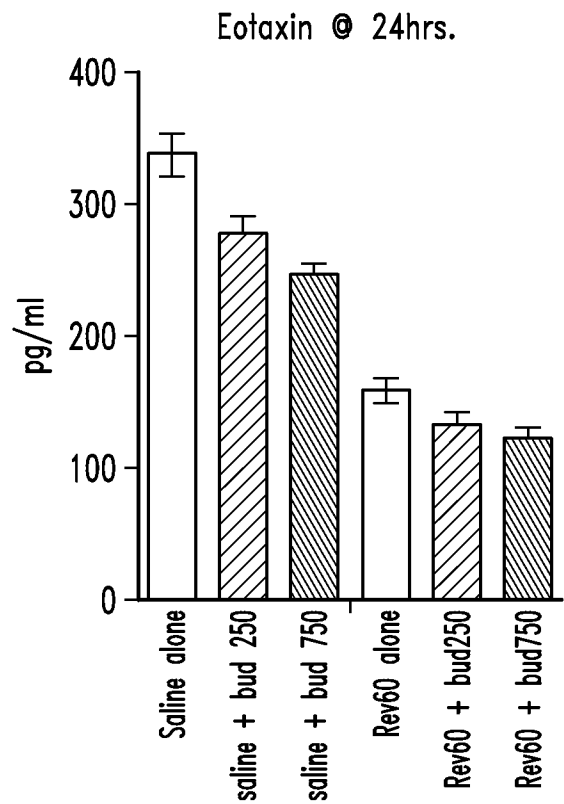
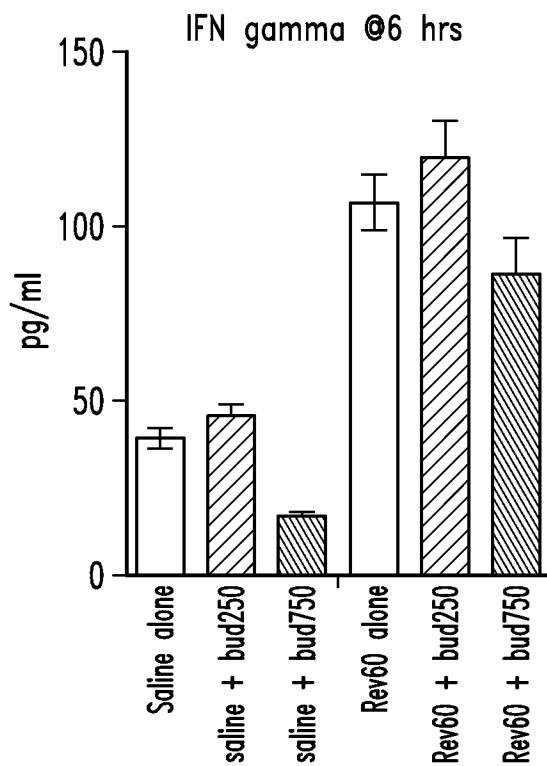
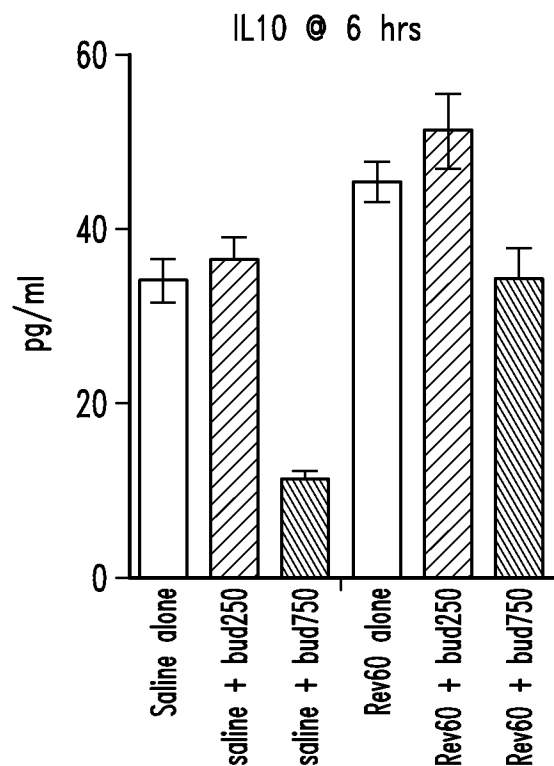
*Fig. 109**Fig. 110*

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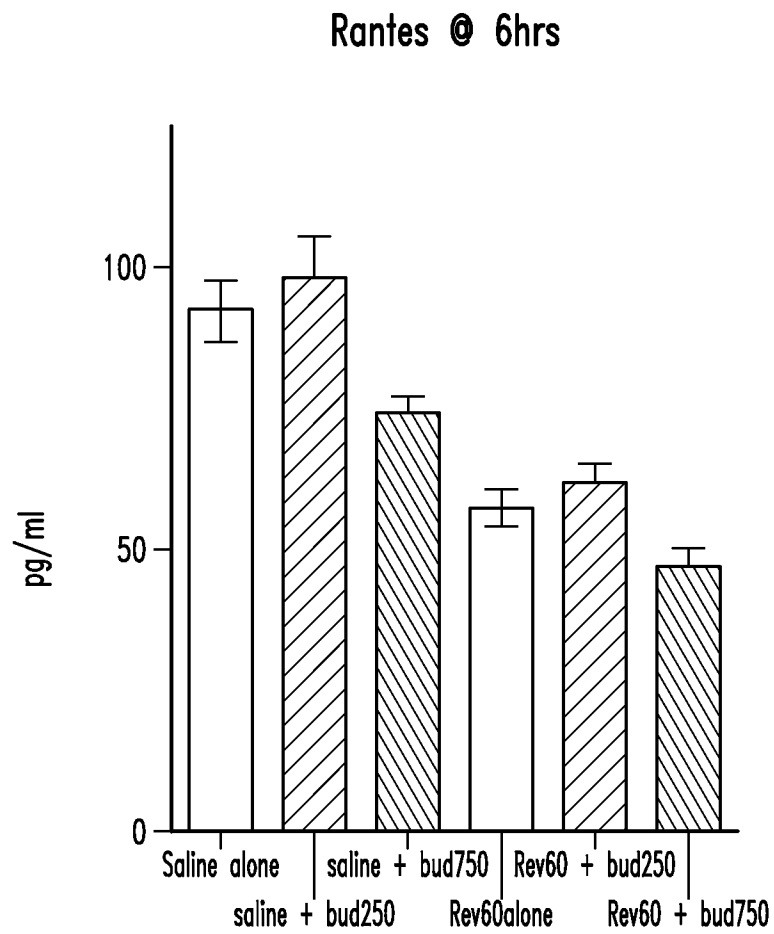
*Fig. 111A**Fig. 111B**Fig. 111C*

*Fig. 112A**Fig. 112B**Fig. 112C*

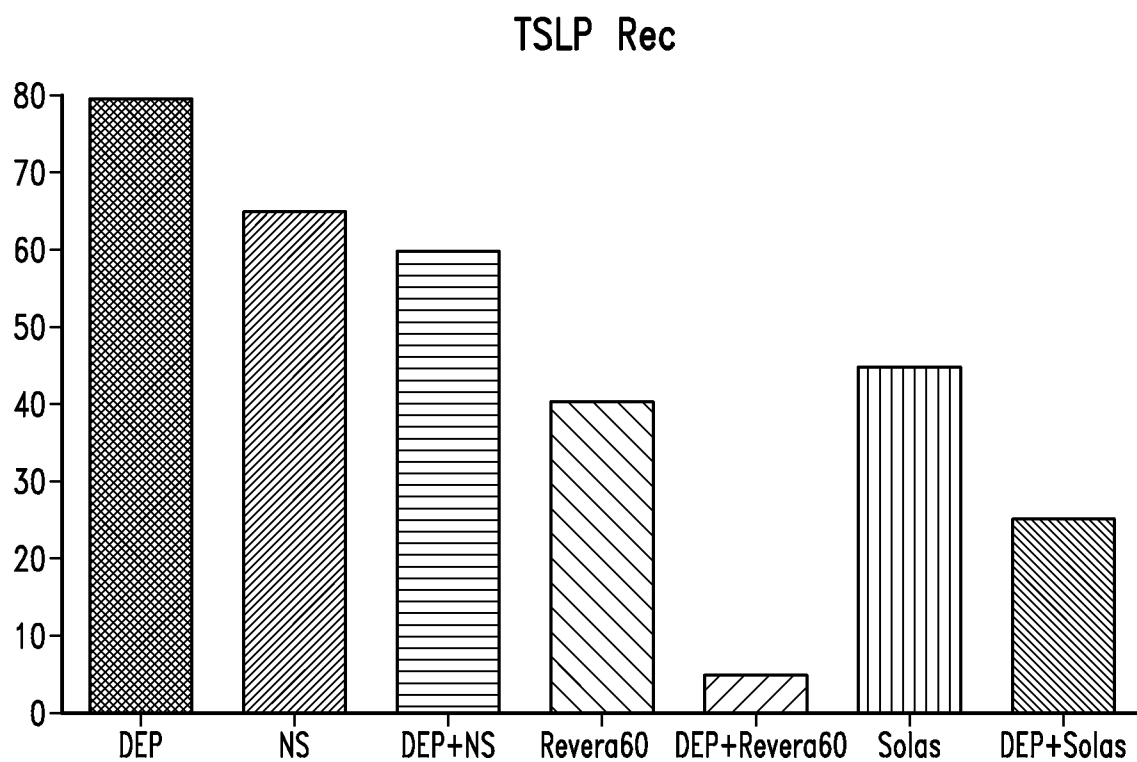
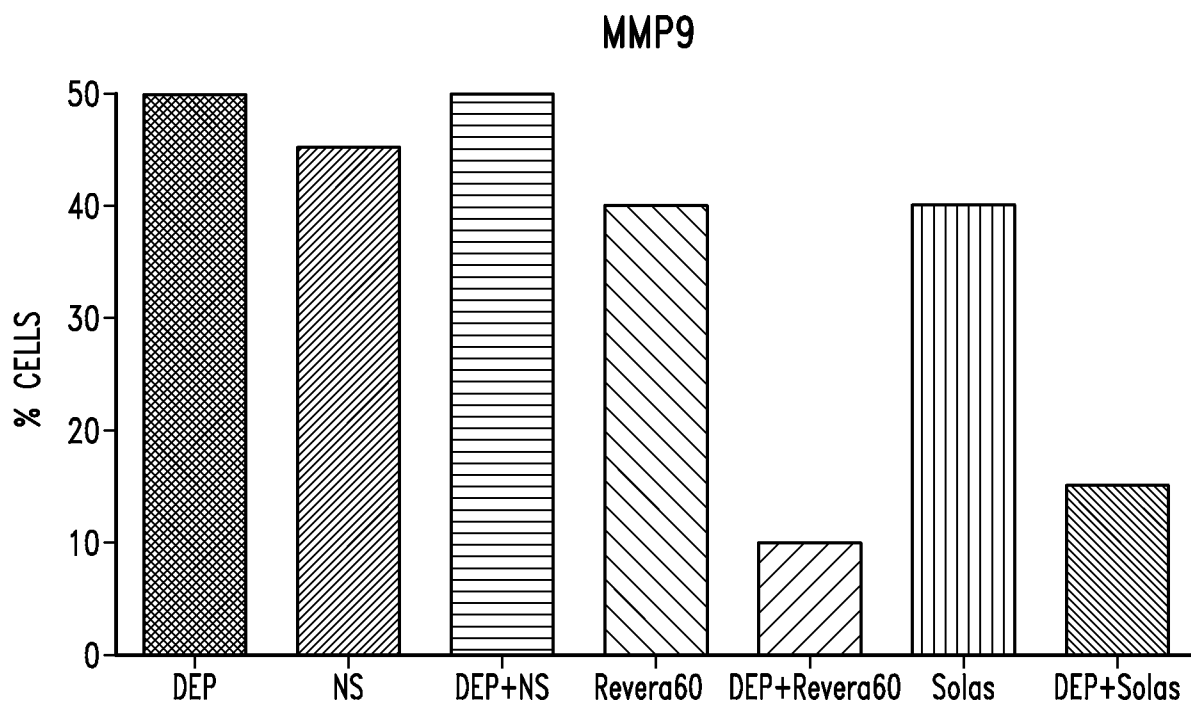
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*Fig. 113A**Fig. 113B**Fig. 113C**Fig. 113D*

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*Fig. 114*

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*Fig. 115**Fig. 116*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/81021

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68; C12N 5/00 (2009.01)

USPC - 422/50; 435/325; 332/106

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 422/50; 435/325; 332/106

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

USPC: 422/50; 435/325; 332/106 (see also text search below)

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

Electronic Databases Searched: pubWEST, USPTO, Google, Answers.com, Google Patents

Search Terms Used: gpcr, g protein, phospholipase C, intracellular, signal, modulation, membrane, inflammation, asthma, neurodegenerative, alzheimer's, aging, hormone, resistance, copd, bronchial, constriction, alveolar, insulin, amd, ti

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2003/0022288 A1 (ZUKER et al.) 30 January 2003 (20.01.2003) entire document especially para [0019], [0022]-[0023], [0028]-[0029], [0032], [0037]-[0038], [0041], [0043], [0046]-[0047], [0052], [0054], [0058]-[0061], [0082], [0107], [0109], [0124], [0127], [0131], [0183], [0186], [0192], [0196]-[0197], [0199]-[0203], [0217], [0220], [0230], [0239] and [0254]	1-8, 11-15, 20 and 24-33 ----- 9-10, 16-19 and 21-23
Y	US 2004/0235732 A1 to (ZHOU et al.) 25 November 2004 (25.11.2004) para [0043]	9-10
Y	US 2005/0096458 A1 (DUMAS et al.) 05 May 2005 (05.05.2005) para [1213], [1218]-[1221] and [1718]	16-18
Y	US 2004/0241154 A1 (DAVIS et al.) 02 December 2004 (02.12.2004) para [0084]-[0086], [0089]	19, 21-22
Y	US 2007/0021331 A1 (FRASER et al.) 25 January 2007 (25.01.2007) para [0088] and [0400]	23

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

01 January 2009 (01.01.2009)

Date of mailing of the international search report

22 JAN 2009

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