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(54) Title: COMPOSITIONS AND METHODS FOR TREATING THYMIC STROMAL LYMPHOPOIETIN (TSLP)-MEDIATED CONDITIONS

(57) Abstract: Provided are methods for treating a TSLP-mediated or TSLPR-mediated disease or condition, comprising administration of an electrokinetically altered aqueous fluid comprising an ionic aqueous solution of charge-stabilized oxygen-containing nanostructures substantially having an average diameter of less than about 100 nanometers and stably configured in the ionic aqueous fluid in an amount sufficient for treating a TSLP-mediated or TSLPR-mediated disease or condition. The charge-stabilized oxygen-containing nanostructures are preferably stably configured in the fluid in an amount sufficient to provide for modulation of cellular membrane potential and/or conductivity. Certain aspects comprising modulation or down-regulation of TSLP expression and/or activity have utility for treating TSLP-mediated or TSLPR-mediated diseases or conditions as disclosed herein (e.g., disorders of the immune system, allergic inflammation, allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, and food allergies, inflammatory arthritis, rheumatoid arthritis, psoriasis, IgE-mediated disorders, and rhino-conjunctivitis).



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COMPOSITIONS AND METHODS FOR TREATING THYMIC STROMAL LYMPHOPOIETIN (TSLP)-MEDIATED CONDITIONS

FIELD OF THE INVENTION

The present invention relates generally to thymic stromal lymphopoietin (TSLP) and
5 TSLP-mediated conditions, and more particularly to TSLP and TSLP receptor-mediated
conditions (e.g., disorders of the immune system, allergic inflammation, allergic airway
inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema,
allergic asthma, asthma, obstructive airways disease, chronic obstructive pulmonary disease, and
food allergies, inflammatory arthritis, rheumatoid arthritis, psoriasis, IgE-mediated disorders,
10 and rhino-conjunctivitis). Particularly preferred aspects relate to modulation (e.g., treating) of
TSLP and TSLP receptor-mediated conditions, 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, and in further combination
therapy aspects with administration of such electrokinetic fluids in combination with at least one
15 additional therapeutic agent.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to United States Provisional Patent
Application Serial Nos. 61/107,480, and 61/107,453, both filed 22 October 2008, and to United
20 States Utility Patent Application Serial No. 12/258,210, filed 24 October 2008, both
incorporated here in by reference in their entirety.

BACKGROUND

Thymic stromal lymphopoietin (TSLP). Thymic stromal lymphopoietin (TSLP) is an
25 IL-7-like cytokine that triggers dendritic cell-mediated Th2-type inflammatory responses and is
considered as a master switch for allergic inflammation. TSLP is an integral growth factor to
both B and T cell development and maturation. Particularly, murine TSLP supports B
lymphopoieses and is required for B cell proliferation. Murine TSLP plays a crucial role in
controlling the rearrangement of the T cell receptor-gamma (TCR γ) locus and has a substantial
30 stimulatory effect on thymocytes and mature T cells. See, for example, Friend et al., *Exp.*
Hematol., 22:321-328, 1994; Ray et al., *Eur. J. Immunol.*, 26:10-16, 1996; Candeias et al.,
Immunology Letters, 57:9-14, 1997.

TSLP possesses cytokine activity similar to IL-7. For instance, TSLP can replace IL-7 in stimulating B cell proliferation responses (Friend et al., supra). Although TSLP and IL-7 mediate similar effects on target cells, they appear to have distinct signaling pathways and likely vary in their biologic response. For Example, although TSLP modulates the activity of STAT5, it fails to activate any Janus family tyrosine kinase members (Levin et. al., *J. Immunol.*, 162:677-683, 1999).

TSLP effects on dendritic cells and TNF production. After human TSLP and the human TSLP receptor were cloned in 2001, it was discovered that human TSLP potently activated immature CD11c+ myeloid dendritic cells (mDCs) (see, e.g., Reche et al., *J. Immunol.*, 167:336-343, 2001 and Soumelis et al., *Nat. Immunol.*, 3:673-680, 2002). Th2 cells are generally defined in immunology textbooks and literature as CD4+ T cells that produce IL-4, IL-5, IL-13, and IL-10. And Th1 cells such as CD4+ T cells produce IFN- γ and sometimes TNF. When TSLP-DCs are used to stimulate naive allogeneic CD4+ T cells in vitro, a unique type of Th2 cell is induced which produces the classical Th2 cytokines IL-4, IL-5, and IL-13, and large amounts of TNF, but little or no IL-10 or interferon- γ (Reche et al., *Supra*) (see also, e.g., Soumelis et al., *Nat. Immunol.*, 3:673-680, 2002). TNF is not typically considered a Th2 cytokine. However, TNF is prominent in asthmatic airways and genotypes that correlate with increased TNF secretion are associated with an increased asthma risk. See Shah et al., *Clin. Exp. Allergy.*, 25:1038-1044, 1995 and Moffatt, M.F. and Cookson, W.O., *Hum. Mol. Genet.*, 6:551-554, 1997.

TSLP induces human mDCs to express the TNF superfamily protein OX40L at both the mRNA and protein level (Ito et al., *J. Exp. Med.*, 202:1213-1223). The expression of OX40L by TSLP-DCs is important for the elaboration of inflammatory Th2 cells. Thus, TSLP-activated DCs create a Th2-permissive microenvironment by up-regulating OX40L without inducing the production of Th1-polarizing cytokines. *Id.*

TSLP expression, allergen-specific responses and asthma. Early studies have shown that TSLP mRNA was highly expressed by human primary skin keratinocytes, bronchial epithelial cells, smooth muscle cells, and lung fibroblasts (Soumelis et al., *Nat. Immunol.*, 3:673-680, 2002). Because TSLP is expressed mainly in keratinocytes of the apical layers of the epidermis, this suggests that TSLP production is a feature of fully differentiated keratinocytes. TSLP expression in patients with atopic dermatitis was associated with Langerhans cell migration and activation in situ which suggests that TSLP may contribute directly to the activation of these cells which could subsequently migrate into the draining lymph nodes and prime allergen-specific responses. *Id.* In a more recent study, it was shown by in situ

hybridization that TSLP expression was increased in asthmatic airways and correlated with both the expression of Th2-attracting chemokines and with disease severity which provided a link between TSLP and asthma (Ying et al., *J. Immunol.*, 174:8183-8190, 2005).

TSLP receptor (TSLPR) and allergic asthma. The TSLP receptor (TSLPR) is approximately 50 kDa protein and has significant similarity to the common γ -chain. TSLPR is a novel type 1 cytokine receptor, which, combined with IL-7Ra (CD127), constitutes a TSLP receptor complex as described, for example, in Pandey et al., *Nat. Immunol.*, 1:59-64, 2000. TSLPR has a tyrosine residue near its carboxyl terminus, which can associate with phosphorylated STAT5 and mediate multiple biological functions when engaged with TSLP (Isaksen et al., *J. Immunol.*, 168:3288-3294, 2002).

Human TSLPR is expressed by monocytes and CD11c+ dendritic cells, and TSLP binding induces the expression of the Th2 cell-attracting chemokines CCL17 and CCL22. Furthermore, as stated above, the TSLPR-induced activation of dendritic cells indirectly results in the increased secretion of Th2 cytokines IL-4, -5 and -13, which may be necessary for the regulation of CD4+ T cell homeostasis. In mice, deficiency of TSLPR has no effect on lymphocyte numbers. However, a deficiency of TSLPR and common γ -chain results in fewer lymphocytes as compared to mice deficient in the common γ -chain alone. See Reche et al., *J. Immunol.*, 167:336-343, 2001 and Soumelis et al., *Nat. Immunol.*, 3:673-680, 2002.

Studies have found that TSLP and the TSLPR play a critical role in the initiation of allergic diseases in mice. In one study, it was demonstrated that mice engineered to overexpress TSLP in the skin developed atopic dermatitis which is characterized by eczematous skin lesions containing inflammatory infiltrates, a dramatic increase in circulating Th2 cells and elevated serum IgE (Yoo et al., *J. Exp. Med.*, 202:541-549, 2005). The study suggested that TSLP may directly activate DCs in mice. In another study, conducted by Li et al., the group confirmed that transgenic mice overexpressing TSLP in the skin developed atopic dermatitis which solidifies the link between TSLP and the development of atopic dermatitis.

Another set of studies demonstrated that TSLP is required for the initiation of allergic airway inflammation in mice in vivo. In one study, Zhou et al. demonstrated that lung specific expression of a TSLP transgene induced allergic airway inflammation (asthma) which is characterized by massive infiltration of leukocytes (including Th2 cells), goblet cell hyperplasia, and subepithelial fibrosis, and increased serum IgE levels (Zhou et al., *Nat. Immunol.*, 6:1047-1053, 2005). However, in contrast, mice lacking the TSLPR failed to develop asthma in response to inhaled antigens (Zhou et al., supra and Al-Shami et al., *J. Exp. Med.*, 202:829-839,

2005). Thus, these studies together demonstrate that TSLP is required for the initiation of allergic airway inflammation in mice.

Further, in a study conducted by Yong-Jun et al., it was demonstrated that epithelial cell-derived TSLP triggers DC-mediated inflammatory Th2 responses in humans which suggest that TSLP represents a master switch of allergic inflammation at the epithelial cell-DC interface (Yong-Jun et al., *J. Exp. Med.*, 203:269-273, 2006).

In a recent study, it was shown that modulation of DCs function by inhibiting TSLPR lessened the severity in mice (Liyun Shi et al., *Clin. Immunol.*, 129:202-210, 2008). In another set of studies, it was demonstrated that TSLPR was not only expressed in DCs, but also on macrophages, mast cells, and CD4+ T cells (Rochman et al., *J. Immunol.*, 178:6720-6724, 2007 and Omori M. and Ziegler S., *J. Immunol.*, 178:1396-1404, 2007). In order to rule out the direct effects of TSLPR neutralization on CD4+ T cells or other effector cells in allergic inflammation, Liyun Shi et al. performed experiments wherein OVA-loaded DCs were in vitro treated with anti-TSLPR before adoptive transfer to the airways of naive mice. It has previously been found that OVA-DCs triggered strong eosinophilic airway inflammation and accompanied with massive production of Th2 cytokines such as IL-4 and IL-5 (Sung et al., *J. Immunol.*, 166:1261-1271 and Lambrecht et al., *J. Clin. Invest.*, 106:551-559, 2000). However, pretreating OVA-DCs with anti-TSLPR resulted in a significant reduction of eosinophils and lymphocyte infiltration as well as IL-4 and IL-5 levels, further illuminating the role that TSLPR plays in DC-primed allergic disease. This result also supports that blocking of TSLPR on DCs will aid in controlling airway inflammation (Liyun Shi et al., *supra*).

There has been a growing body of experiments implicating the role of TSLP/TSLPR in various physiological and pathological processes. Physiological roles of TSLP include modulating the immune system, particularly in stimulating B and T cell proliferation, development, and maturation. TSLP plays a vital role in the pathobiology of allergic asthma and local antibody mediated blockade of TSLP receptor function to alleviate allergic diseases. Thus, interplay between TSLP and TSLP receptor is believed to be important in many physiological disease processes such as: allergic inflammation, skin lesions of patients with atopic dermatitis or atopic eczema, allergic asthma and asthma.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 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

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effect. Additionally, the non-electrokinetic control pressure pot fluid PP60 resulted in approximately 50% reduction in DEP induced TSLP receptor expression.

Figure 2 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. Additionally, the non-electrokinetic control pressure pot fluid PP60 resulted in approximately 30% reduction in DEP-induced cell surface attached MMP9 levels.

Figures 3 A-C demonstrate the results of a series of patch clamping experiments that assessed the effects of the electrokinetically generated fluid (*e.g.*, RNS-60 and Solas) on epithelial cell membrane polarity and ion channel activity at two time-points (15 min (left panels) and 2 hours (right panels)) and at different voltage protocols.

Figures 4 A-C show, in relation to the experiments relating to Figures 3 A-C, the graphs resulting from the subtraction of the Solas current data from the RNS-60 current data at three voltage protocols (A. stepping from zero mV; B. stepping from -60 mV; C. stepping from -120 mV) and the two time-points (15 mins (open circles) and 2 hours (closed circles)).

Figures 5 A-D demonstrate the results of a series of patch clamping experiments that assessed the effects of the electrokinetically generated fluid (*e.g.*, Solas (panels A. and B.) and RNS-60 (panels C. and D.)) on epithelial cell membrane polarity and ion channel activity using different external salt solutions and at different voltage protocols (panels A. and C. show stepping from zero mV; panels B. and D. show stepping from -120 mV).

Figures 6 A-D show, in relation to the experiments relating to Figures 5 A-D, the graphs resulting from the subtraction of the CsCl current data (shown in Figure 5) from the 20 mM CaCl₂ (diamonds) and 40 mM CaCl₂ (filled squares) current data at two voltage protocols (panels A. and C. stepping from zero mV; B. and D. stepping from -120 mV) for Solas (panels A. and B.) and Revera 60 (panels C. and D.).

Figures 7A and B demonstrate the results of a patch clamp experiment that assessed the effects of diluting the electrokinetically generated fluid (*e.g.*, RNS-60) on epithelial cell membrane polarity and ion channel activity.

SUMMARY OF EXEMPLARY EMBODIMENTS

In accordance with one embodiment of the present invention, there is provided a

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method for treating a TSLP-mediated or TSLPR-mediated disease or condition, comprising administration to a mammal in need thereof, a therapeutically effective amount of an ionic aqueous solution of charge-stabilized oxygen-containing nanobubbles having an average diameter of less than 100 nanometers sufficient for treating a TSLP-mediated or TSLPR-mediated disease or condition, wherein the TSLP-mediated or TSLPR-mediated disease or condition of the immune system comprises at least one selected from the group consisting of allergic inflammation and inflammatory arthritis. In certain aspects, the charge-stabilized oxygen-containing nanostructures are stably configured in the ionic aqueous fluid in an amount sufficient to provide, upon contact of a living cell by the fluid, modulation of at least one of cellular membrane potential and cellular membrane conductivity.

In certain method aspects, the charge-stabilized oxygen-containing nanostructures are the major charge-stabilized gas-containing nanostructure species in the fluid. In particular embodiments, the percentage of dissolved oxygen molecules present in the fluid as the charge-stabilized oxygen-containing nanostructures is a percentage selected from the group consisting of greater than: 0.01%, 0.1%, 1%, 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; and 95%. In particular aspects, the total dissolved oxygen is substantially present in the charge-stabilized oxygen-containing nanostructures. In certain embodiments, the charge-stabilized oxygen-containing nanostructures substantially have an average diameter of less than a size selected from the group consisting of: 90 nm; 80 nm; 70 nm; 60 nm; 50 nm; 40 nm; 30 nm; 20 nm; 10 nm; and less than 5 nm.

In particular method aspects, the ionic aqueous solution comprises a saline solution. In certain aspects, the electrokinetically-altered aqueous fluid is superoxygenated.

In particular method aspects, the electrokinetically-altered aqueous fluid comprises a form of solvated electrons.

In certain aspects, alteration of the electrokinetically-altered aqueous fluid comprises exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects. In certain embodiments, exposure to the localized electrokinetic effects comprises 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 aspects, the TSLP-mediated or TSLPR-mediated disease or condition comprises a disease or disorder of the immune system, including but not limited to allergic inflammation. In particular aspects, the allergic inflammation comprises at least one of allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, IgE-mediated disorders, rhino-conjunctivitis and food allergies. In certain embodiments, the TSLP-mediated or TSLPR-mediated disease or condition comprises inflammatory arthritis, for example comprising at least one of rheumatoid arthritis and psoriasis.

In certain aspects, the method further comprises combination therapy, wherein at least one additional therapeutic agent is administered to the patient. In particular embodiments, the at least one additional therapeutic agent is selected from the group consisting of short-acting β_2 -agonists, long-acting β_2 -agonists, anticholinergics, corticosteroids, systemic corticosteroids, mast cell stabilizers, leukotriene modifiers, methylxanthines, and combinations thereof. In certain aspects, the at least one additional therapeutic agent is selected from the group consisting of: bronchodilators consisting of β_2 -agonists including albuterol, levalbuterol, pirbuterol, arformoterol, formoterol, salmeterol, and anticholinergics such as ipratropium and tiotropium; corticosteroids including beclomethasone, budesonide, flunisolide, fluticasone, mometasone, triamcinolone, methylprednisolone, prednisolone, prednisone; leukotriene modifiers including montelukast, zafirlukast, and zileuton; mast cell stabilizers including cromolyn and nedocromil; methylxanthines including theophylline, combination drugs including ipratropium and albuterol, fluticasone and salmeterol, budesonide and formoterol; antihistamines including hydroxyzine, diphenhydramine, loratadine, cetirizine, and hydrocortisone; immune system modulating drugs including tacrolimus and pimecrolimus; cyclosporine; azathioprine; mycophenolatemofetil; and combinations thereof. In particular aspects, the at least one additional therapeutic agent is a TSLP and/or TSLPR antagonist, and in particular embodiments, the TSLP and/or TSLPR antagonist is selected from the group consisting of neutralizing antibodies specific for TSLP and the TSLP receptor, soluble TSLP receptor molecules, and TSLP receptor fusion proteins, including TSLPR-immunoglobulin Fc molecules or polypeptides that encode components of more than one receptor chain.

In particular method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises altering at least one of cellular membrane structure or function comprising altering at least one of a conformation, ligand binding activity, and a catalytic activity of a membrane associated protein or constituent. 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 certain embodiments, the transmembrane receptor comprises a G-Protein Coupled Receptor (GPCR). In particular aspects, the G-Protein Coupled Receptor (GPCR) interacts with a G protein a subunit, for example, 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 $G_{\alpha_{12}}$, and in certain embodiments the at least one G protein a subunit is G_{α_q} .

In particular method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulating whole-cell conductance, for example,

wherein modulating whole-cell conductance comprises modulating at least one of a linear and a non-linear voltage-dependent contribution of the whole-cell conductance.

In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of a calcium dependant cellular messaging pathway or system. In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of phospholipase C activity. In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of adenylate cyclase (AC) activity. In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of intracellular signal transduction associated with at least one condition or symptom selected from the group consisting of diseases or disorders of the immune system, allergic inflammation, allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, IgE-mediated disorders, rhino-conjunctivitis, food allergies, inflammatory arthritis, rheumatoid arthritis and psoriasis.

Particular method aspects comprise administration of the electrokinetic fluid to a cell network or layer, and further comprise modulation of an intercellular junction therein. In certain embodiments, the intracellular junction comprises at least one selected from the group consisting of tight junctions, gap junctions, zona adherens and desmosomes. In particular aspects, the cell network or layers comprise at least one selected from the group consisting of pulmonary epithelium, bronchial epithelium, and intestinal epithelium.

In certain method aspects, the electrokinetically altered aqueous fluid is oxygenated, wherein the oxygen in the fluid is present in an amount of at least 8 ppm, 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 method aspects, the electrokinetically altered aqueous fluid comprises at least one of solvated electrons, and electrokinetically modified or charged oxygen species, for example, wherein the form of 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. In certain aspects, the electrokinetically altered aqueous fluid comprises a form of solvated electrons stabilized by molecular oxygen.

In certain aspects, the ability of the electrokinetically-altered fluid to modulate at least one of cellular membrane potential and cellular membrane conductivity persists for at least two,

at least three, at least four, at least five, at least 6, at least 12 months, or longer periods, in a closed gas-tight container.

In certain aspects, the amount of oxygen present in charge-stabilized oxygen-containing nanostructures of the electrokinetically-altered fluid is at least 8 ppm, at least 15, ppm, at least 20 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 particular aspects, treating comprises administration by at least one of topical, inhalation, intranasal, and intravenous.

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DETAILED DESCRIPTION

Provided are methods for treating a TSLP-mediated or TSLPR-mediated disease or condition, comprising administration of an electrokinetically altered aqueous fluid comprising an ionic aqueous solution of charge-stabilized oxygen-containing nanostructures substantially having an average diameter of less than about 100 nanometers and stably configured in the ionic aqueous fluid in an amount sufficient for treating a TSLP-mediated or TSLPR-mediated disease or condition. The charge-stabilized oxygen-containing nanostructures are preferably stably configured in the fluid in an amount sufficient to provide for modulation of cellular membrane potential and/or conductivity. Certain aspects comprising modulation or down-regulation of TSLP expression and/or activity have utility for treating TSLP-mediated or TSLPR-mediated diseases or conditions as disclosed herein (*e.g.*, disorders of the immune system, allergic inflammation, allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, and food allergies, inflammatory arthritis, rheumatoid arthritis, psoriasis, IgE-mediated disorders, and rhino-conjunctivitis).

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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).

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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, the electrokinetically altered aqueous fluid comprise an ionic aqueous solution of charge-stabilized oxygen-containing nanostructures substantially having an average diameter of less than about 100 nanometers and stably configured in the ionic aqueous fluid in an amount sufficient to provide, upon contact of a living cell by the fluid, modulation of at least one of cellular membrane potential and cellular membrane conductivity.

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., $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, 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 herein.

In particular aspects, the electrokinetically altered aqueous fluids display synergy with Albuterol, and with Budesonide as shown 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 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.,
5 closed gas-tight container).

Therefore, further aspects provide said electrokinetically-generated solutions and methods of producing an electrokinetically altered oxygenated aqueous fluid or solution, comprising: providing a flow of a fluid material between two spaced surfaces in relative motion and defining a mixing volume therebetween, wherein the dwell time of a single pass of the
10 flowing fluid material within and through the mixing volume is greater than 0.06 seconds or greater than 0.1 seconds; and introducing oxygen (O₂) into the flowing fluid material within the mixing volume under conditions suitable to dissolve at least 20 ppm, at least 25 ppm, at least 30, at least 40, at least 50, or at least 60 ppm oxygen into the material, and electrokinetically alter the fluid or solution. In certain aspects, the oxygen is infused into the material in less than 100
15 milliseconds, less than 200 milliseconds, less than 300 milliseconds, or less than 400 milliseconds. In particular embodiments, the ratio of surface area to the volume is at least 12, at least 20, at least 30, at least 40, or at least 50.

Yet further aspects, provide a method of producing an electrokinetically altered oxygenated aqueous fluid or solution, comprising: providing a flow of a fluid material between
20 two spaced surfaces defining a mixing volume therebetween; and introducing oxygen into the flowing material within the mixing volume under conditions suitable to infuse at least 20 ppm, at least 25 ppm, at least 30, at least 40, at least 50, or at least 60 ppm oxygen into the material in less than 100 milliseconds, less than 200 milliseconds, less than 300 milliseconds, or less than 400 milliseconds. In certain aspects, the dwell time of the flowing material within the mixing
25 volume is greater than 0.06 seconds or greater than 0.1 seconds. In particular embodiments, the ratio of surface area to the volume is at least 12, at least 20, at least 30, at least 40, or at least 50.

In particular aspects the administered inventive electrokinetically-altered fluids comprise charge-stabilized oxygen-containing nanostructures in an amount sufficient to provide modulation of at least one of cellular membrane potential and cellular membrane conductivity.
30 In certain embodiments, the electrokinetically-altered fluids are superoxygenated (e.g., RNS-20, RNS-40 and RNS-60, comprising 20 ppm, 40 ppm and 60 ppm dissolved oxygen, respectively, in standard saline). In particular embodiments, the electrokinetically-altered fluids are not-superoxygenated (e.g., RNS-10 or Solas, comprising 10 ppm (e.g., approx. ambient levels of dissolved oxygen in standard saline). In certain aspects, the salinity, sterility, pH, etc., of the

inventive electrokinetically-altered fluids is established at the time of electrokinetic production of the fluid, and the sterile fluids are administered by an appropriate route. Alternatively, at least one of the salinity, sterility, pH, etc., of the fluids is appropriately adjusted (e.g., using sterile saline or appropriate diluents) to be physiologically compatible with the route of administration prior to administration of the fluid. Preferably, and diluents and/or saline solutions and/or buffer compositions used to adjust at least one of the salinity, sterility, pH, etc., of the fluids are also electrokinetic fluids, or are otherwise compatible.

In particular aspects, the inventive electrokinetically-altered fluids comprise saline (e.g., one or more dissolved salt(s); e.g., alkali metal based salts (Li, Na, K, Rb, Cs, etc.), alkaline earth based salts (e.g., Mg, Ca), etc., transition metal-based salts (e.g., Cr, Fe, Co, Ni, Cu, Zn, etc.), along with any suitable anion/counterion components). Particular aspects comprise mixed salt based electrokinetic fluids (e.g., Na, K, Ca, Mg, etc., in various combinations and concentrations). In particular aspects, the inventive electrokinetically-altered fluids comprise standard saline (e.g., approx. 0.9% NaCl, or about 0.15 M NaCl). In particular aspects, the inventive electrokinetically-altered fluids comprise saline at a concentration of at least 0.0002 M, at least 0.0003 M, at least 0.001 M, at least 0.005 M, at least 0.01 M, at least 0.015 M, at least 0.1 M, at least 0.15 M, or at least 0.2 M. In particular aspects, the conductivity of the inventive electrokinetically-altered fluids is at least 10 μ S/cm, at least 40 μ S/cm, at least 80 μ S/cm, at least 100 μ S/cm, at least 150 μ S/cm, at least 200 μ S/cm, at least 300 μ S/cm, or at least 500 μ S/cm, at least 1 mS/cm, at least 5 mS/cm, 10 mS/cm, at least 40 mS/cm, at least 80 mS/cm, at least 100 mS/cm, at least 150 mS/cm, at least 200 mS/cm, at least 300 mS/cm, or at least 500 mS/cm. In particular aspects, any salt may be used in preparing the inventive electrokinetically-altered fluids, provided that they allow for formation of biologically active salt-stabilized nanostructures (e.g., salt-stabilized oxygen-containing nanostructures) as disclosed herein.

According to particular aspects, the biological effects of the inventive fluid compositions comprising charge-stabilized gas-containing nanostructures can be modulated (e.g., increased, decreased, tuned, etc.) by altering the ionic components of the fluids as, for example, described above, and/or by altering the gas component of the fluid. In preferred aspects, oxygen is used in preparing the inventive electrokinetic fluids. In additional aspects mixtures of oxygen along with at least one other gas selected from Nitrogen, Oxygen, Argon, Carbon dioxide, Neon, Helium, krypton, hydrogen and Xenon.

Exemplary preferred embodiments:

Particular aspect provide a method for treating a TSLP-mediated or TSLPR-mediated disease or condition, comprising administration to a mammal in need thereof, a therapeutically effective amount of an electrokinetically altered aqueous fluid comprising an ionic aqueous solution of charge-stabilized oxygen-containing nanostructures substantially having an average diameter of less than about 100 nanometers and stably configured in the ionic aqueous fluid in an amount sufficient for treating a TSLP-mediated or TSLPR-mediated disease or condition. In certain aspects, the charge-stabilized oxygen-containing nanostructures are stably configured in the ionic aqueous fluid in an amount sufficient to provide, upon contact of a living cell by the fluid, modulation of at least one of cellular membrane potential and cellular membrane conductivity.

In certain method aspects, the charge-stabilized oxygen-containing nanostructures are the major charge-stabilized gas-containing nanostructure species in the fluid. In particular embodiments, the percentage of dissolved oxygen molecules present in the fluid as the charge-stabilized oxygen-containing nanostructures is a percentage selected from the group consisting of greater than: 0.01%, 0.1%, 1%, 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; and 95%. In particular aspects, the total dissolved oxygen is substantially present in the charge-stabilized oxygen-containing nanostructures. In certain embodiments, the charge-stabilized oxygen-containing nanostructures substantially have an average diameter of less than a size selected from the group consisting of: 90 nm; 80 nm; 70 nm; 60 nm; 50 nm; 40 nm; 30 nm; 20 nm; 10 nm; and less than 5 nm.

In particular method aspects, the ionic aqueous solution comprises a saline solution. In certain aspects, the electrokinetically-altered aqueous fluid is superoxygenated.

In particular method aspects, the electrokinetically-altered aqueous fluid comprises a form of solvated electrons.

In certain aspects, alteration of the electrokinetically-altered aqueous fluid comprises exposure of the fluid to hydrodynamically-induced, localized electrokinetic effects. In certain embodiments, exposure to the localized electrokinetic effects comprises 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 aspects, the TSLP-mediated or TSLPR-mediated disease or condition comprises a disease or disorder of the immune system, including but not limited to allergic inflammation. In particular aspects, the allergic inflammation comprises at least one of allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic

eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, IgE-mediated disorders, rhino-conjunctivitis and food allergies. In certain embodiments, the TSLP-mediated or TSLPR-mediated disease or condition comprises inflammatory arthritis, for example comprising at least one of rheumatoid arthritis and psoriasis.

5 In certain aspects, the method further comprises combination therapy, wherein at least one additional therapeutic agent is administered to the patient. In particular embodiments, the at least one additional therapeutic agent is selected from the group consisting of short-acting β_2 -agonists, long-acting β_2 -agonists, anticholinergics, corticosteroids, systemic corticosteroids, mast cell stabilizers, leukotriene modifiers, methylxanthines, and combinations thereof. In
10 certain aspects, the at least one additional therapeutic agent is selected from the group consisting of: bronchodilators consisting of β_2 -agonists including albuterol, levalbuterol, pirbuterol, arformoterol, formoterol, salmeterol, and anticholinergics such as ipratropium and tiotropium; corticosteroids including beclomethasone, budesonide, flunisolide, fluticasone, mometasone, triamcinolone, methylprednisolone, prednisolone, prednisone; leukotriene modifiers including
15 montelukast, zafirlukast, and zileuton; mast cell stabilizers including cromolyn and nedocromil; methylxanthines including theophylline, combination drugs including ipratropium and albuterol, fluticasone and salmeterol, budesonide and formoterol; antihistamines including hydroxyzine, diphenhydramine, loratadine, cetirizine, and hydrocortisone; immune system modulating drugs including tacrolimus and pimecrolimus; cyclosporine; azathioprine; mycophenolatemofetil; and
20 combinations thereof. In particular aspects, the at least one additional therapeutic agent is a TSLP and/or TSLPR antagonist, and in particular embodiments, the TSLP and/or TSLPR antagonist is selected from the group consisting of neutralizing antibodies specific for TSLP and the TSLP receptor, soluble TSLP receptor molecules, and TSLP receptor fusion proteins, including TSLPR-immunoglobulin Fc molecules or polypeptides that encode components of
25 more than one receptor chain.

In particular method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises altering at least one of cellular membrane structure or function comprising altering at least one of a conformation, ligand binding activity, and a catalytic activity of a membrane associated protein or constituent. In certain aspects, the
30 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 certain embodiments, the transmembrane receptor comprises a G-Protein Coupled Receptor (GPCR). In particular aspects, the G-Protein Coupled Receptor (GPCR) interacts with a G protein a subunit, for example, 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 $G_{\alpha_{12}}$, and in certain embodiments the at least one G protein subunit is G_{α_q} .

In particular method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulating whole-cell conductance, for example, wherein modulating whole-cell conductance comprises modulating at least one of a linear and a non-linear voltage-dependent contribution of the whole-cell conductance.

In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of a calcium dependant cellular messaging pathway or system. In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of phospholipase C activity. In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of adenylate cyclase (AC) activity. In certain method aspects, modulation of at least one of cellular membrane potential and cellular membrane conductivity comprises modulation of intracellular signal transduction associated with at least one condition or symptom selected from the group consisting of diseases or disorders of the immune system, allergic inflammation, allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, IgE-mediated disorders, rhinoconjunctivitis, food allergies, inflammatory arthritis, rheumatoid arthritis and psoriasis.

Particular method aspects comprise administration of the electrokinetic fluid to a cell network or layer, and further comprise modulation of an intercellular junction therein. In certain embodiments, the intracellular junction comprises at least one selected from the group consisting of tight junctions, gap junctions, zona adherens and desmosomes. In particular aspects, the cell network or layers comprise at least one selected from the group consisting of pulmonary epithelium, bronchial epithelium, and intestinal epithelium.

In certain method aspects, the electrokinetically altered aqueous fluid is oxygenated, wherein the oxygen in the fluid is present in an amount of at least 8 ppm, 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 method aspects, the electrokinetically altered aqueous fluid comprises at least one of solvated electrons, and electrokinetically modified or charged oxygen species, for example, wherein the form of 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. In certain aspects, the electrokinetically altered aqueous fluid comprises a form of solvated electrons stabilized by molecular oxygen.

In certain aspects, the ability of the electrokinetically-altered fluid to modulate at least one of cellular membrane potential and cellular membrane conductivity persists for at least two, at least three, at least four, at least five, at least 6, at least 12 months, or longer periods, in a closed gas-tight container.

In certain aspects, the amount of oxygen present in charge-stabilized oxygen-containing nanostructures of the electrokinetically-altered fluid is at least 8 ppm, at least 15, ppm, at least 20 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 particular aspects, treating comprises administration by at least one of topical, inhalation, intranasal, and intravenous.

Exemplary relevant Molecular Interactions:

Conventionally, quantum properties are thought to belong to elementary particles of less than 10^{-10} meters, while the macroscopic world of our everyday life is referred to as classical, in that it behaves according to Newton's laws of motion.

Recently, molecules have been described as forming clusters that increase in size with dilution. These clusters measure several micrometers in diameter, and have been reported to increase in size non-linearly with dilution. Quantum coherent domains measuring 100 nanometers in diameter have been postulated to arise in pure water, and collective vibrations of water molecules in the coherent domain may eventually become phase locked to electromagnetic field fluctuations, providing for stable oscillations in water, providing a form of 'memory' in the form of excitation of long lasting coherent oscillations specific to dissolved substances in the water that change the collective structure of the water, which may in turn determine the specific coherent oscillations that develop. Where these oscillations become stabilized by magnetic field phase coupling, the water, upon dilution may still carry 'seed' coherent oscillations. As a cluster of molecules increases in size, its electromagnetic signature is correspondingly amplified, reinforcing the coherent oscillations carried by the water.

Despite variations in the cluster size of dissolved molecules and detailed microscopic structure of the water, a specificity of coherent oscillations may nonetheless exist. One model for considering changes in properties of water is based on considerations involved in crystallization.

A simplified protonated water cluster forming a nanoscale cage is shown in Applicants' previous patent application: WO 2009/055729. A protonated water cluster typically takes the form of $H^+(H_2O)_n$. Some protonated water clusters occur naturally, such as in the ionosphere. Without being bound by any particular theory, and according to particular aspects, other types of water clusters or structures (clusters, nanocages, etc) are possible, including structures comprising oxygen and stabilized electrons imparted to the inventive output materials. Oxygen atoms may be caught in the resulting structures. The chemistry of the semi-bound nanocage allows the oxygen and/or stabilized electrons to remain dissolved for extended periods of time. Other atoms or molecules, such as medicinal compounds, can be caged for sustained delivery purposes. The specific chemistry of the solution material and dissolved compounds depend on the interactions of those materials.

Fluids processed by the mixing device have been shown previously via experiments to exhibit different structural characteristics that are consistent with an analysis of the fluid in the context of a cluster structure. See, for example, WO 2009/055729.

Charge-stabilized nanostructures (e.g., charge stabilized oxygen-containing nanostructures):

As described previously in Applicants' WO 2009/055729, "Double Layer Effect," "Dwell Time," "Rate of Infusion," and "Bubble size Measurements," the electrokinetic mixing device creates, in a matter of milliseconds, a unique non-linear fluid dynamic interaction of the first material and the second material with complex, dynamic turbulence providing complex mixing in contact with an effectively enormous surface area (including those of the device and of the exceptionally small gas bubbles of less than 100 nm) that provides for the novel electrokinetic effects described herein. Additionally, feature-localized electrokinetic effects (voltage/current) were demonstrated using a specially designed mixing device comprising insulated rotor and stator features.

As well-recognized in the art, charge redistributions and/or solvated electrons are known to be highly unstable in aqueous solution. According to particular aspects, Applicants' electrokinetic effects (e.g., charge redistributions, including, in particular aspects, solvated electrons) are surprisingly stabilized within the output material (e.g., saline solutions, ionic solutions). In fact, as described herein, the stability of the properties and biological activity of the inventive electrokinetic fluids (e.g., RNS-60 or Solas) can be maintained for months in a gas-tight container, indicating involvement of dissolved gas (e.g., oxygen) in helping to generate and/or maintain, and/or mediate the properties and activities of the inventive solutions. Significantly, the charge redistributions and/or solvated electrons are stably configured in the inventive electrokinetic ionic aqueous fluids in an amount sufficient to provide, upon contact

with a living cell (e.g., mammalian cell) by the fluid, modulation of at least one of cellular membrane potential and cellular membrane conductivity (see, e.g., cellular patch clamp working Example 23 from WO 2009/055729 and as disclosed herein).

As described herein under "Molecular Interactions," to account for the stability and biological compatibility of the inventive electrokinetic fluids (e.g., electrokinetic saline solutions), Applicants have proposed that interactions between the water molecules and the molecules of the substances (e.g., oxygen) dissolved in the water change the collective structure of the water and provide for nanoscale cage clusters, including nanostructures comprising oxygen and/or stabilized electrons imparted to the inventive output materials. Without being bound by mechanism, the configuration of the nanostructures in particular aspects is such that they: comprise (at least for formation and/or stability and/or biological activity) dissolved gas (e.g., oxygen); enable the electrokinetic fluids (e.g., RNS-60 or Solas saline fluids) to modulate (e.g., impart or receive) charges and/or charge effects upon contact with a cell membrane or related constituent thereof; and in particular aspects provide for stabilization (e.g., carrying, harboring, trapping) solvated electrons in a biologically-relevant form.

According to particular aspects, and as supported by the present disclosure, in ionic or saline (e.g., standard saline, NaCl) solutions, the inventive nanostructures comprise charge stabilized nanostructures (e.g., average diameter less than 100 nm) that may comprise at least one dissolved gas molecule (e.g., oxygen) within a charge-stabilized hydration shell. According to additional aspects, the charge-stabilized hydration shell may comprise a cage or void harboring the at least one dissolved gas molecule (e.g., oxygen). According to further aspects, by virtue of the provision of suitable charge-stabilized hydration shells, the charge-stabilized nanostructure and/or charge-stabilized oxygen containing nano-structures may additionally comprise a solvated electron (e.g., stabilized solvated electron).

Without being bound by mechanism or particular theory, after the present priority date, charge-stabilized microbubbles stabilized by ions in aqueous liquid in equilibrium with ambient (atmospheric) gas have been proposed (Bunkin et al., *Journal of Experimental and Theoretical Physics*, 104:486-498, 2007; incorporated herein by reference in its entirety). According to particular aspects of the present invention, Applicants' novel electrokinetic fluids comprise a novel, biologically active form of charge-stabilized oxygen-containing nanostructures, and may further comprise novel arrays, clusters or associations of such structures.

According to the charge-stabilized microbubble model, the short-range molecular order of the water structure is destroyed by the presence of a gas molecule (e.g., a dissolved gas molecule initially complexed with a nonadsorptive ion provides a short-range order defect),

providing for condensation of ionic droplets, wherein the defect is surrounded by first and second coordination spheres of water molecules, which are alternately filled by adsorptive ions (e.g., acquisition of a 'screening shell of Na^+ ions to form an electrical double layer) and nonadsorptive ions (e.g., Cl^- ions occupying the second coordination sphere) occupying six and
5 12 vacancies, respectively, in the coordination spheres. In under-saturated ionic solutions (e.g., undersaturated saline solutions), this hydrated 'nucleus' remains stable until the first and second spheres are filled by six adsorptive and five nonadsorptive ions, respectively, and then undergoes Coulomb explosion creating an internal void containing the gas molecule, wherein the adsorptive ions (e.g., Na^+ ions) are adsorbed to the surface of the resulting void, while the
10 nonadsorptive ions (or some portion thereof) diffuse into the solution (Bunkin et al., *supra*). In this model, the void in the nanostructure is prevented from collapsing by Coulombic repulsion between the ions (e.g., Na^+ ions) adsorbed to its surface. The stability of the void-containing nanostructures is postulated to be due to the selective adsorption of dissolved ions with like charges onto the void/bubble surface and diffusive equilibrium between the dissolved gas and
15 the gas inside the bubble, where the negative (outward electrostatic pressure exerted by the resulting electrical double layer provides stable compensation for surface tension, and the gas pressure inside the bubble is balanced by the ambient pressure. According to the model, formation of such microbubbles requires an ionic component, and in certain aspects collision-mediated associations between particles may provide for formation of larger order clusters
20 (arrays) (*Id.*).

The charge-stabilized microbubble model suggests that the particles can be gas microbubbles, but contemplates only spontaneous formation of such structures in ionic solution in equilibrium with ambient air, is uncharacterized and silent as to whether oxygen is capable of forming such structures, and is likewise silent as to whether solvated electrons might be
25 associated and/or stabilized by such structures.

According to particular aspects, the inventive electrokinetic fluids comprising charge-stabilized nanostructures and/or charge-stabilized oxygen-containing nanostructures are novel and fundamentally distinct from the postulated non-electrokinetic, atmospheric charge-stabilized microbubble structures according to the microbubble model. Significantly, this conclusion is
30 unavoidable, deriving, at least in part, from the fact that control saline solutions do not have the biological properties disclosed herein, whereas Applicants' charge-stabilized nanostructures provide a novel, biologically active form of charge-stabilized oxygen-containing nanostructures.

According to particular aspects of the present invention, Applicants' novel electrokinetic device and methods provide for novel electrokinetically-altered fluids comprising significant

quantities of charge-stabilized nanostructures in excess of any amount that may or may not spontaneously occur in ionic fluids in equilibrium with air, or in any non-electrokinetically generated fluids. In particular aspects, the charge-stabilized nanostructures comprise charge-stabilized oxygen-containing nanostructures. In additional aspects, the charge-stabilized nanostructures are all, or substantially all charge-stabilized oxygen-containing nanostructures, or the charge-stabilized oxygen-containing nanostructures the major charge-stabilized gas-containing nanostructure species in the electrokinetic fluid.

According to yet further aspects, the charge-stabilized nanostructures and/or the charge-stabilized oxygen-containing nanostructures may comprise or harbor a solvated electron, and thereby provide a novel stabilized solvated electron carrier. In particular aspects, the charge-stabilized nanostructures and/or the charge-stabilized oxygen-containing nanostructures provide a novel type of electride (or inverted electride), which in contrast to conventional solute electrides having a single organically coordinated cation, rather have a plurality of cations stably arrayed about a void or a void containing an oxygen atom, wherein the arrayed sodium ions are coordinated by water hydration shells, rather than by organic molecules. According to particular aspects, a solvated electron may be accommodated by the hydration shell of water molecules, or preferably accommodated within the nanostructure void distributed over all the cations. In certain aspects, the inventive nanostructures provide a novel 'super electride' structure in solution by not only providing for distribution/stabilization of the solvated electron over multiple arrayed sodium cations, but also providing for association or partial association of the solvated electron with the caged oxygen molecule(s) in the void—the solvated electron distributing over an array of sodium atoms and at least one oxygen atom. According to particular aspects, therefore, 'solvated electrons' as presently disclosed in association with the inventive electrokinetic fluids, may not be solvated in the traditional model comprising direct hydration by water molecules. Alternatively, in limited analogy with dried electride salts, solvated electrons in the inventive electrokinetic fluids may be distributed over multiple charge-stabilized nanostructures to provide a 'lattice glue' to stabilize higher order arrays in aqueous solution.

In particular aspects, the inventive charge-stabilized nanostructures and/or the charge-stabilized oxygen-containing nanostructures are capable of interacting with cellular membranes or constituents thereof, or proteins, etc., to mediate biological activities. In particular aspects, the inventive charge-stabilized nanostructures and/or the charge-stabilized oxygen-containing nanostructures harboring a solvated electron are capable of interacting with cellular membranes or constituents thereof, or proteins, etc., to mediate biological activities.

In particular aspects, the inventive charge-stabilized nanostructures and/or the charge-stabilized oxygen-containing nanostructures interact with cellular membranes or constituents thereof, or proteins, etc., as a charge and/or charge effect donor (delivery) and/or as a charge and/or charge effect recipient to mediate biological activities. In particular aspects, the inventive charge-stabilized nanostructures and/or the charge-stabilized oxygen-containing nanostructures harboring a solvated electron interact with cellular membranes as a charge and/or charge effect donor and/or as a charge and/or charge effect recipient to mediate biological activities.

In particular aspects, the inventive charge-stabilized nanostructures and/or the charge-stabilized oxygen-containing nanostructures are consistent with, and account for the observed stability and biological properties of the inventive electrokinetic fluids, and further provide a novel electride (or inverted electride) that provides for stabilized solvated electrons in aqueous ionic solutions (e.g., saline solutions, NaCl, etc.).

In particular aspects, the charge-stabilized oxygen-containing nanostructures substantially comprise, take the form of, or can give rise to, charge-stabilized oxygen-containing nanobubbles. In particular aspects, charge-stabilized oxygen-containing clusters provide for formation of relatively larger arrays of charge-stabilized oxygen-containing nanostructures, and/or charge-stabilized oxygen-containing nanobubbles or arrays thereof. In particular aspects, the charge-stabilized oxygen-containing nanostructures can provide for formation of hydrophobic nanobubbles upon contact with a hydrophobic surface.

In particular aspects, the charge-stabilized oxygen-containing nanostructures substantially comprise at least one oxygen molecule. In certain aspects, the charge-stabilized oxygen-containing nanostructures substantially comprise at least 1, at least 2, at least 3, at least 4, at least 5, at least 10 at least 15, at least 20, at least 50, at least 100, or greater oxygen molecules. In particular aspects, charge-stabilized oxygen-containing nanostructures comprise or give rise to nanobubbles (e.g., hydrophobic nanobubbles) of about 20 nm x 1.5 nm, comprise about 12 oxygen molecules (e.g., based on the size of an oxygen molecule (approx 0.3 nm by 0.4 nm), assumption of an ideal gas and application of $n=PV/RT$, where $P=1$ atm, $R=0.082057$ l.atm/mol.K; $T=295$ K; $V=\pi r^2 h=4.7 \times 10^{-22}$ L, where $r=10 \times 10^{-9}$ m, $h=1.5 \times 10^{-9}$ m, and $n=1.95 \times 10^{-22}$ moles).

In certain aspects, the percentage of oxygen molecules present in the fluid that are in such nanostructures, or arrays thereof, having a charge-stabilized configuration in the ionic aqueous fluid is a percentage amount selected from the group consisting of greater than: 0.1%, 1%; 2%; 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; and greater than 95%. Preferably, this percentage is greater than about 5%,

greater than about 10%, greater than about 15%, or greater than about 20%. In additional aspects, the substantial size of the charge-stabilized oxygen-containing nanostructures, or arrays thereof, having a charge-stabilized configuration in the ionic aqueous fluid is a size selected from the group consisting of less than: 100 nm; 90 nm; 80 nm; 70 nm; 60 nm; 50 nm; 40 nm; 30 nm; 20 nm; 10 nm; 5 nm; 4 nm; 3 nm; 2 nm; and 1 nm. Preferably, this size is less than about 50 nm, less than about 40 nm, less than about 30 nm, less than about 20 nm, or less than about 10 nm.

In certain aspects, the inventive electrokinetic fluids comprise solvated electrons. In further aspects, the inventive electrokinetic fluids comprises charge-stabilized nanostructures and/or charge-stabilized oxygen-containing nanostructures, and/or arrays thereof, which comprise at least one of: solvated electron(s); and unique charge distributions (polar, symmetric, asymmetric charge distribution). In certain aspects, the charge-stabilized nanostructures and/or charge-stabilized oxygen-containing nanostructures, and/or arrays thereof, have paramagnetic properties.

By contrast, relative to the inventive electrokinetic fluids, control pressure pot oxygenated fluids (non-electrokinetic fluids) and the like do *not* comprise such electrokinetically generated charge-stabilized biologically-active nanostructures and/or biologically-active charge-stabilized oxygen-containing nanostructures and/or arrays thereof, capable of modulation of at least one of cellular membrane potential and cellular membrane conductivity.

Systems for Making Gas-Enriched Fluids

The presently disclosed system and methods allow gas (e.g. oxygen) to be enriched stably at a high concentration with minimal passive loss. This system and methods can be effectively used to enrich a wide variety of gases at heightened percentages into a wide variety of fluids. By way of example only, deionized water at room temperature that typically has levels of about 2-3 ppm (parts per million) of dissolved oxygen can achieve levels of dissolved oxygen ranging from at least about 5 ppm, at least about 10 ppm, at least about 15 ppm, at least about 20 ppm, at least about 25 ppm, at least about 30 ppm, at least about 35 ppm, at least about 40 ppm, at least about 45 ppm, at least about 50 ppm, at least about 55 ppm, at least about 60 ppm, at least about 65 ppm, at least about 70 ppm, at least about 75 ppm, at least about 80 ppm, at least about 85 ppm, at least about 90 ppm, at least about 95 ppm, at least about 100 ppm, or any value greater or therebetween using the disclosed systems and/or methods. In accordance with a particular exemplary embodiment, oxygen-enriched water may be generated with levels of about 30-60 ppm of dissolved oxygen.

Table 1 illustrates various partial pressure measurements taken in a healing wound treated with an oxygen-enriched saline solution (Table 1) and in samples of the gas-enriched oxygen-enriched saline solution of the present invention.

5

TABLE 1

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	

TSLP and TSLP-mediated conditions

TSLP and TSLPR agonists/antagonists: An agent that has affinity for and stimulates physiologic activity at cell receptors normally stimulated by naturally occurring substances, thus triggering a biochemical response. A TSLP receptor agonist has affinity for the TSLP receptor and stimulates an activity induced by the binding of TSLP with its receptor. For example, a TSLP/TSLP receptor agonist is a molecule that binds to the TSLP receptor and induces intracellular signaling. In contrast, an “antagonist” is an agent that inhibits activity of a cell receptor normally stimulated by a naturally occurring substance. Accordingly, a TSLP/TSLP receptor antagonist binds to TSLP or to the TSLP receptor and inhibits binding of TSLP to the TSLP receptor and/or inhibits an activity normally induced by binding of TSLP with its receptor. For example, a TSLP/TSLP receptor antagonist can bind to TSLP or to the TSLP receptor and diminish or prevent binding, for example, by blocking binding, of TSLP to the TSLP receptor. Alternatively, a TSLP/TSLP receptor antagonist can bind to the TSLP receptor and diminish or prevent downstream signaling that would normally be induced by the binding of TSLP with its receptor. Agonists and antagonists can include a variety of classes of molecules including polypeptides, such as ligand-like polypeptides, antibodies, and fragments or subsequences thereof. Agonists and antagonists can also include fusion polypeptides, antibodies, peptides (such as peptides of less than about 20 amino acids in length), and small molecules. Exemplary antagonists include: neutralizing antibodies specific for TSLP and the TSLP receptor, soluble TSLP receptor molecules, and TSLP receptor fusion proteins, such as TSLPR-

immunoglobulin Fc molecules or polypeptides that encode components of more than one receptor chain, that thereby mimic a physiological receptor heterodimer or higher order oligomer. If the receptor includes more than one polypeptide chain, a single chain fusion can be utilized.

5 Antibody: A polypeptide ligand comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope (e.g., as an antigen, such as TSLP or a fragment thereof, or a TSLP receptor or a fragment thereof). This includes intact immunoglobulins and the variants and portions of them well known in the art, such as Fab' fragments, F(ab)'.sub.2 fragments, single chain Fv proteins ("scFv"), and disulfide
10 stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFvs, the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies (e.g., humanized murine antibodies), heteroconjugate antibodies (e.g.,
15 bispecific antibodies). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, Ill.); Kuby, J., Immunology, 3rd Ed., W.H. Freeman & Co., New York, 1997. Typically, an immunoglobulin has a heavy and a light chain. Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains"). In combination, the heavy and the light chain variable regions specifically bind the antigen. Light
20 and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs". The extent of the framework region and CDRs has been defined (see, Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Department of Health and Human Services, 1991, which is hereby incorporated by reference). The Kabat database is now maintained online. The
25 sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space. Antibodies include monoclonal antibodies, humanized antibodies, etc.

 TSLP antagonists include small molecule antagonists, antibodies to TSLP, antibodies to
30 the TSLP receptor, and TSLP receptor fusion proteins, such as TSLPR-immunoglobulin Fc molecules or polypeptides that encode components of more than one receptor chain, that thereby mimic a physiological receptor heterodimer or higher order oligomer, amongst others. TSLP has been shown to bind directly to a type I cytokine receptor superfamily member (which are also known as hematopoietin receptor superfamily members), TSLPR. TSLPR has been cloned. The

functional high-affinity receptor for TSLP has been demonstrated to include two polypeptides, TSLPR and the IL-7 receptor alpha chain. Thus, both TSLP and IL-7 shares IL-7Ralpha as a component of their receptors. However, these receptors are distinctive in that the TSLP receptor additionally contains TSLPR whereas the IL-7 receptor additionally contains the common
5 cytokine receptor gamma chain, which is a signal-transducing component of various cytokine receptors. TSLPR (and Fc fusions of this receptor chain) are described, for example, in Published U.S. Patent Application No. 2002/0160949, which is incorporated herein by reference.

Antibodies to TSLP polypeptides are known in the art. In addition, anti-TSLPR
10 antibodies are commercially available (R & D Systems, Minneapolis, Minn., cat. no. MAB981; DNAX Research, Inc., Palo Alto, Calif.). Antibodies are also prepared against TSLP receptor or TSLP by immunization with specified epitopes, such as regions of increased antigenicity determined by the Welling plot of Vector NTI.RTM. Suite (Informax, Inc, Bethesda, Md.). The sequence of the TSLP receptor, and regions of increased antigenicity in human TSLP receptor
15 are disclosed in U.S. Patent Publication No. 2003/0186875. Pharmaceutical compositions (see above) generally include a therapeutically effective amount of a TSLP antagonist, and can also include additional agents. The preparation of pharmaceutical compositions is disclosed above.

Indications

TSLP and TSLPR are believed to have roles in many types of allergic conditions
20 including, but not limited to, disorders of the immune system, allergic inflammation, allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, allergic asthma, asthma, obstructive airways disease, chronic obstructive pulmonary disease (COPD), food allergies, inflammatory arthritis, rheumatoid arthritis, psoriasis, IgE-mediated disorders, and rhino-conjunctivitis. TSLP involvement in DC-mediated inflammatory
25 Th2 responses has been shown in several publications including a recent review by Ziegler and Liu (Ziegler and Liu, *Nat. Immunol.*, 7:709-714, 2005, which is incorporated by reference in its entirety).

Allergic airway inflammation. A recent study demonstrated that TSLP is required for the initiation of allergic airway inflammation in mice in vivo (Zhou et al., *Nat. Immunol.*, 6:1047-
30 1053, 2005). In this study, Zhou et al. demonstrated that lung specific expression of a TSLP transgene induced allergic airway inflammation (asthma) which is characterized by massive infiltration of leukocytes (including Th2 cells), goblet cell hyperplasia, and subepithelial fibrosis, and increased serum IgE levels. In addition, a recent study showed that allergen challenge caused a rapid accumulation of TSLP in the airways of asthmatic mice (Liyun Shi et

al., *Clin. Immunol.*, 129:202-210, 2008). These results indicate that TSLP plays an important role in the pathogenesis of allergic airway inflammation. Here Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP. According to certain embodiments, the inventive electrokinetically-altered fluids, have substantial utility for treating allergic airway inflammation and similar conditions.

Allergic inflammation. A recent review summarizes and describes the results from studies investigating the role of TSLP in allergic inflammation e.g. allergic skin inflammation. (Ziegler and Liu, 2008). In particular, studies have shown that normal skin or nonlesional skin in patients with atopic dermatitis has no detectable TSLP protein, whereas, the skin taken from acute and chronic atopic dermatitis lesions has high expression of TSLP. In another study, mice lacking TSLPR were constructed and examined for effects on allergic skin inflammation. (He et al., PNAS, 105:11875-11880, 2008, which is incorporated by reference in its entirety). He et al., discovered that skin inflammation due to an allergen in mice lacking TSLPR was significantly reduced than when compared to wildtype. In yet another study, it was demonstrated that mice engineered to overexpress TSLP in the skin developed atopic dermatitis which is characterized by eczematous skin lesions containing inflammatory infiltrates, a dramatic increase in circulating Th2 cells and elevated serum IgE (Yoo et al., *J. Exp. Med.*, 202:541-549, 2005). The study suggested that TSLP may directly activate DCs in mice. In another study, conducted by Li et al., the group confirmed that transgenic mice overexpressing TSLP in the skin developed atopic dermatitis which solidifies the link between TSLP and the development of atopic dermatitis. These results indicate that TSLP plays an important role in the pathogenesis of allergic inflammation, e.g. allergic skin inflammation (e.g., atopic dermatitis and eczema). Here Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP. According to certain embodiments, the inventive electrokinetically-altered fluids, have substantial utility for treating allergic inflammation, e.g. allergic skin inflammation (e.g., atopic dermatitis and eczema) and similar conditions.

Psoriasis. A recent study showed that TSLP had substantially higher expression in skin biopsies taken from patients with acute psoriasis (Guttman-Yassky, et al., *J. Allergy and Clinical Immunology* 119:1210-1217, 2007). This result indicates that TSLP plays an important role in the pathogenesis of psoriasis. Herein, Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP. According to certain embodiments, therefore, the inventive electrokinetically-altered fluids, have substantial utility for treating psoriasis and similar conditions.

Allergic asthma. Recently, a study showed that allergen challenge caused a rapid accumulation of TSLP in the airways of asthmatic mice. (Liyun Shi et al., *Clin. Immunol.*, 129:202-210, 2008). In the same study, it was shown that modulation of DCs function by inhibiting TSLPR lessened the severity in mice. These results indicate that TSLP plays an important role in the pathogenesis of allergic asthma. Herein, Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP. According to certain embodiments, therefore, the inventive electrokinetically-altered fluids, have substantial utility for treating allergic asthma and similar conditions.

Obstructive airways disease. A recent study demonstrated that COPD is associated with elevated bronchial mucosal expression of TSLP (Ying et al., *J Immunol*, 181:2790-2798, 2008). COPD is a type of obstructive airways diseases. This results indicate that TSLP plays an important role in the pathogenesis of obstructive airways disease, e.g. COPD. Herein, Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP. According to certain embodiments, therefore, the inventive electrokinetically-altered fluids, have substantial utility for treating obstructive airways disease, e.g. COPD.

Food allergies. Dendritic cells of the intestines were found to stimulate naïve T cells, skewing them to a TH2 response in an OX40L dependent manner. (Blazquez AB, Berin MC. Gastrointestinal dendritic cells promote Th2 skewing via OX40L. *J Immunol*, 180:4441-4450, 2008, which is incorporated by reference in its entirety). In addition, a recent review discusses the presence of TSLP in the intestines and its role in regulation of immune homeostasis. (Iliev ID, Matteoli G, Rescigno M. The yin and yang of intestinal epithelial cells in controlling dendritic cell function. *J Exp Med*; 204:2253-2257, 2007, which is incorporated by reference in its entirety). These results indicate that TSLP has a role in food allergies. Herein, Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP. According to certain embodiments, therefore, the inventive electrokinetically-altered fluids, have substantial utility for treating food allergies and similar conditions.

Inflammatory arthritis. A recent study found increased levels of TSLP in synovial fluid specimens derived from patients with rheumatoid arthritis (RA) when compared with synovial fluid obtained from patients with other forms of arthritis. (Koyama et al. *Biochem and Biophys Res Comm.*, 357:99-104, 2007, which is incorporated by reference in its entirety). The same study found that use of an anti-TSLP neutralizing antibody ameliorated a TNF- α -dependent experimental arthritis induced by anti-type II collagen antibody in mice. These results indicate that TSLP is a significant player in inflammatory arthritis such as RA. Herein, Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP. According

to certain embodiments, therefore, the inventive electrokinetically-altered fluids, have substantial utility for treating inflammatory arthritis and similar conditions, e.g. RA.

5 *Allergic rhinitis.* In a recent research study, Mou et al., discovered that TSLP was present at both the mRNA and protein levels in the nasal mucosa of all patients tested that were suffering from allergic rhinitis (AR). (Mou et al., *Acta Oto-laryngologica*, 129:297-301, 2009, which is incorporated by reference in its entirety). In addition, TSLP levels were tightly correlated with the severity of AR. These results indicate that TSLP plays an important role in the pathogenesis of AR and/or rhino-conjunctivitis. Herein, Applicants show that the inventive electrokinetically-altered fluids significantly downregulated TSLP in a relevant model system.

10 According to certain embodiments, therefore, the inventive electrokinetically-altered fluids, have substantial utility for treating AR, allergic rhino-conjunctivitis and similar conditions.

Methods of Treatment

 The term “treating” refers to, and includes, reversing, alleviating, inhibiting the progress of, or preventing a disease, disorder or condition, or one or more symptoms thereof; and

15 “treatment” and “therapeutically” refer to the act of treating, as defined herein.

 A “therapeutically effective amount” is any amount of any of the compounds utilized in the course of practicing the invention provided herein that is sufficient to reverse, alleviate, inhibit the progress of, or prevent a disease, disorder or condition, or one or more symptoms thereof.

20 Certain embodiments herein relate to therapeutic compositions and methods of treatment for a subject by preventing or alleviating at least one symptom of inflammation associated with certain conditions or diseases. Many conditions or diseases associated with inflammation disorders have been treated with steroids, methotrexate, immunosuppressive drugs including cyclophosphamide, cyclosporine, azathioprine and leflunomide, nonsteroidal anti-inflammatory

25 agents such as aspirin, acetaminophen and COX-2 inhibitors, gold agents and anti-malarial treatments.

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.

30 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 inflammation. 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.

5 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.

10 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
15 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,
20 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
25 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,
30 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, irritation 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.

- 5 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
10 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
15 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
20 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,
25 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
30 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, 5 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 10 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 15 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, 20 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 25 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 30 accompany the administration of the therapeutic composition and the desired physiological effect.

It will be appreciated that the compounds of the combination may be administered: (1) simultaneously by combination of the compounds in a co-formulation or (2) by alternation, i.e. delivering the compounds serially, sequentially, in parallel or simultaneously in separate

pharmaceutical formulations. In alternation therapy, the delay in administering the second, and optionally a third active ingredient, should not be such as to lose the benefit of a synergistic therapeutic effect of the combination of the active ingredients. According to certain embodiments by either method of administration (1) or (2), ideally the combination should be administered to achieve the most efficacious results. In certain embodiments by either method of administration (1) or (2), ideally the combination should be administered to achieve peak plasma concentrations of each of the active ingredients. A one pill once-per-day regimen by administration of a combination co-formulation may be feasible for some patients suffering from inflammatory neurodegenerative diseases. According to certain embodiments effective peak plasma concentrations of the active ingredients of the combination will be in the range of approximately 0.001 to 100 μ M. Optimal peak plasma concentrations may be achieved by a formulation and dosing regimen prescribed for a particular patient. It will also be understood that the inventive fluids and a glucocorticoid steroid (e. g., Budesonide) or the physiologically functional derivatives of any thereof, whether presented simultaneously or sequentially, may be administered individually, in multiples, or in any combination thereof. In general, during alternation therapy (2), an effective dosage of each compound is administered serially, where in co-formulation therapy (1), effective dosages of two or more compounds are administered together.

The combinations of the invention may conveniently be presented as a pharmaceutical formulation in a unitary dosage form. A convenient unitary dosage formulation contains the active ingredients in any amount from 1 mg to 1 g each, for example but not limited to, 10 mg to 300 mg. The synergistic effects of the inventive fluid in combination with, for example, a glucocorticoid steroid (e. g., Budesonide) may be realized over a wide ratio, for example 1:50 to 50:1 (inventive fluid: a glucocorticoid steroid (e. g., Budesonide)). In one embodiment the ratio may range from about 1:10 to 10:1. In another embodiment, the weight/weight ratio of inventive fluid to a glucocorticoid steroid (e. g., Budesonide) in a co-formulated combination dosage form, such as a pill, tablet, caplet or capsule will be about 1, i.e. an approximately equal amount of inventive fluid and a glucocorticoid steroid (e. g., Budesonide). In other exemplary co-formulations, there may be more or less inventive fluid and a glucocorticoid steroid (e. g., Budesonide). In one embodiment, each compound will be employed in the combination in an amount at which it exhibits anti-inflammatory activity when used alone. Other ratios and amounts of the compounds of said combinations are contemplated within the scope of the invention.

A unitary dosage form may further comprise inventive fluid and, for example, a glucocorticoid steroid (e. g., Budesonide), or physiologically functional derivatives of either thereof, and a pharmaceutically acceptable carrier.

5 It will be appreciated by those skilled in the art that the amount of active ingredients in the combinations of the invention required for use in treatment will vary according to a variety of factors, including the nature of the condition being treated and the age and condition of the patient, and will ultimately be at the discretion of the attending physician or health care practitioner. The factors to be considered include the route of administration and nature of the formulation, the animal's body weight, age and general condition and the nature and severity of
10 the disease to be treated.

It is also possible to combine any two of the active ingredients in a unitary dosage form for simultaneous or sequential administration with a third active ingredient. The three-part combination may be administered simultaneously or sequentially. When administered sequentially, the combination may be administered in two or three administrations. According to
15 certain embodiments the three-part combination of inventive fluid and a glucocorticoid steroid (e. g., Budesonide) may be administered in any order.

According to particular aspects, the inventive electrokinetically-altered fluids, have substantial utility for treating the TSLP and/or TSLPR-mediated conditions, including but not limited to the exemplary genus of indications disclosed herein. According to additional aspects,
20 the inventive electrokinetically-altered fluids, have utility for treating various subgenera of the exemplary genus, wherein at least one indication of the genus is excluded from each of said subgenera.

EXAMPLE 1

25 *(Synergistic effects of inventive electrokinetically-altered fluids and Albuterol were demonstrated)*

Overview. The inventive electrokinetically-altered fluids provided 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)) and thus
30 provides for a decrease in a patient's albuterol usage. The results disclosed in this Example are also disclosed in Applicants' WO 2009/055729.

First experiment. In a first 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. The results indicate that 50% of the Group B animals were protected from bronchoconstriction out to 10 hours (at which time the test was terminated).

Second experiment. 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 and methods. Guinea Pigs (*Cavia porcellus*) were Hartley albino, Crl:(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. 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.

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 (500µg/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 microsyringe. 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 microsyringe 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 (500 µg/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 a transient increase in penes.

Once prior to test article administration (Day -1) and again at 2, 6, 10, 14, 18, 22 and 26 hours post-dose, 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 parameters 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 bronchoconstriction during the predose methacholine challenge were replaced. Any animal displaying at least 75% of their baseline Penh 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).

Results. The results from this experiment showed that 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, 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, 20 ppm, 40 ppm and 60 ppm) 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. Applicants also showed 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, 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 2

(Effects of inventive electrokinetically-altered fluids on cytokine expression were determined)

Overview. The inventive electrokinetically-altered fluids lowered the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, and GM-CSF), chemokines (IL-8, MIP-1α, 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) when compared to control fluid and increased anti-inflammatory cytokines (e.g., IL1R-a, TIMPs) when compared to control fluid. The results disclosed in this Example are also disclosed in Applicants' WO 2009/055729.

5 In particular aspects, human mixed lymphocytes were stimulated with T3 antigen or PHA in Revalesio 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-a, RANTES, Leptin, TNF- β , TFG- β , and NGF were evaluated. As was shown, pro-inflammatory cytokines (IL-1 β , TNF-a, IL-6, and GM-
10 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.

15 Additionally, 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.

20 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
25 RDC1676-00 (sterile saline processed through the Revalesio proprietary device), and RDC1676-01 (sterile saline processed through the Revalesio proprietary 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
30 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.

5 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.

10 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
15 expression known to be associated with lung inflammation in this model. A unilateral lavage technique was being 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-
20 800 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
25 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
30 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 TRIzol™ or PAXgene™. 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 PAXgene™, 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.

Results from Blood Samples. 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 Revalerio treatment; followed by saline challenged saline treatment; and saline challenged Revalerio 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.

Applicants determined that the level of RANTES (IL-8 super family) produced after treatment with the inventive electrokinetically-altered fluids was less than that produced by the saline only exposed groups. Applicants demonstrated that the inventive electrokinetically-altered fluids caused MCP-1 to be produced at lower levels when compared to that which was produced by the saline only exposed groups. Applicants determined that the level of TNF alpha

produced after treatment with the inventive electrokinetically-altered fluids was less than that produced by the saline only exposed groups.

In addition, Applicants demonstrated that the level of MIP-1 alpha produced after treatment with the inventive electrokinetically-altered fluids was less than that produced by the saline only exposed groups. Applicants demonstrated that the inventive electrokinetically-altered fluids caused IL-1 alpha to be produce at lower levels when compared to that which was produced by the saline only exposed groups. Applicants observed that the level of Vcam produced after treatment with the inventive electrokinetically-altered fluids was less than that produced by the saline only exposed groups. Applicants observed that the level of IL-1 beta produced after treatment with the inventive electrokinetically-altered fluids was less than that produced by the saline only exposed groups. Applicants demonstrated that the inventive electrokinetically-altered fluids caused Eotaxin and MCP-3 to be produce at lower levels when compared to that which was produced by the saline only exposed 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 3

(Effects of the inventive electrokinetically-altered fluids to modulate T-cell proliferation were determined)

Overview. The inventive electrokinetically-altered fluids improved regulatory T-cell function as shown by relatively decreased proliferation. The results disclosed in this Example are also disclosed in Applicants' WO 2009/055729.

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),
5 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
10 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
15 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
20 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 PhosFlowTM buffer and permeabilized in denaturing Permbuffer III[®]. Cells were then stained with antibodies against each particular selected
25 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
30 statistically significant via the Spearman coefficient if the r value was greater than 0.7 or less than -0.7 (two-tailed).

Results. Regulatory T cell proliferation was studied by stimulating cells with diesel exhaust particulate matter (PM, from EPA). Applicants determined that the cells stimulated with PM (no Rev, no Solas) 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. Note that treatment with Rev alone resulted in higher IL-10 levels relative to Saline and Media controls. Similar results were obtained with GITR, Granzyme A, XCL1, pStat5, and Foxp3, respectively.

Applicants also obtained 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 Solas). Treatment with Rev alone resulted in lower tryptase levels relative to Saline and Media controls.

In summary, Applicants observed a decreased proliferation in the presence of PM and Rev relative to PM in control fluid (no Rev, no Solas), indicating that the inventive electrokinetically generated fluid Rev improved regulatory T-cell function as shown by relatively decreased proliferation in the assay. Moreover, the evidence indicates that beta blockade, GPCR blockade and Ca channel blockade affects the activity of Rev on Treg function.

EXAMPLE 4

(Synergistic effects between the inventive electrokinetically-altered fluids and Budesonide were determined)

Overview. The inventive electrokinetically-altered fluids provided for synergistic anti-inflammatory effects with Budesonide in vivo in an art-recognized animal model for allergic asthma. The results disclosed in this Example are also disclosed in Applicants' WO 2009/055729.

Applicants initially performed experiments 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, 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. Additionally, while treatment with the inventive fluid “RDC1676-03” alone 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). Similarly, the Eosinophil % also reflected a similar trend. While RDC1676-03 or Budesonide 750 ug/kg alone did not have a significant effect on Eosinophil % count in the challenged animals, the two in combination reduced the Eosinophil % significantly.

Therefore, Applicants determined, according to particular aspects, that the inventive electrokinetically generated fluids (e.g., RDC1676-03) 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:

Applicants also demonstrated 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.

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

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. 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.

Applicants observed that Rev 60 (or RDC1676-03) alone lowered the blood level of eotaxin significantly at both 6 and 24 hours post challenge. Budesonide 750 ug/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).

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 Interferon gamma are also significantly enhanced at 6 hours after challenge as a result of treatment with Rev 60 alone or in combination with Budesonide. Applicants observed such effects on Interferon gamma and IL 10, respectively. 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 ug/kg 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).

5 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
10 for eosinophils.

Applicants observed that 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 ug/kg or 750 ug/kg. Once again, there was a clear synergistic effect of Budesonide 750 ug/kg and Rev 60. A similar downward trend was observed for a number of
15 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 5

20 *(Effects of the inventive electrokinetically-altered fluids on intercellular tight junctions were determined)*

Overview. The inventive electrokinetically-altered fluids were shown to modulate intercellular tight junctions. The results disclosed in this Example are also disclosed in Applicants' WO 2009/055729.

25 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
30 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-TeflonTM or PolytronTM homogenizer. Samples were stored at -80°C.

Results:

Enhancement of tight junctions. Applicants observed 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. Applicants showed upregulation of the junction adhesion molecules JAM 2 and 3, GJA1, 3, 4 and 5 (junctional adherens), OCLN (occludin), claudins (e.g., CLDN 3, 5, 7, 8, 9, 10), TJP1 (tight junction protein 1), respectively.

EXAMPLE 6

(Effects of the inventive electrokinetically-altered fluids on whole-cell conductance were determined)

Overview. The inventive electrokinetically-altered fluids decreased the whole-cell conductance as demonstrated by patch clamp analysis conducted on bronchial epithelial cells (BEC). Patch clamp analysis conducted on bronchial epithelial cells (BEC) perfused with inventive electrokinetically-altered fluid (RNS-60) revealed that exposure to RNS-60 resulted in a decrease in whole cell conductance. In addition, 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. The results disclosed in this Example are also disclosed in Applicants’ WO 2009/055729.

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. Applicants showed that 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, Applicants showed in the context of T-regulatory cells stimulated with particulate matter (PM), that there was a decreased proliferation of T-regulatory cells in the presence of PM and Rev relative to PM in control fluid (no Rev, no Solas), 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 indicate that beta blockade, GPCR blockade and Ca channel blockade affects the activity of the electrokinetically generated fluids on Treg and PBMC function. Furthermore, Applicants demonstrated, according to additional aspects, upon expose to the inventive fluids, tight junction related proteins were upregulated in lung tissue. Applicants showed upregulation of the junction adhesion molecules JAM 2 and 3, GJA1,3,4 and 5 (junctional adherens), 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").

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:

Applicants determined whole-cell currents under basal (no cAMP) conditions, with a protocol stepping from zero mV holding potential to ± 100 mV. Representative tracings (control, followed by the whole-cell tracings while perfusing the test solution) were made on an average of $n=12$ cells. Composite 'delta' tracings, obtained by subtraction of the test average values, from those under control conditions were obtained. The whole-cell conductance, obtained from the current-to-voltage relationships was 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) was also linear, and the reversal potential was near zero mV. There was a decrease in the whole cell conductance under hyperpolarizing conditions.

In addition, Applicant determined whole-cell currents under basal conditions, with a protocol stepping from -40 mV holding potential to ± 100 mV. Representative tracings (control, followed by the whole-cell tracings while perfusing the test solution) were made on an average of $n=12$ cells. Composite delta tracings were obtained by subtraction of the test average values,

from those under control conditions. The whole-cell conductance obtained from the current-to-voltage relationships was highly linear under both conditions, and reflected 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) was also linear, and the reversal potential was near zero mV. Values were comparatively similar to those obtained with the zero mV protocol.

Applicants determined whole-cell currents under basal conditions, with a protocol stepping from -60 mV holding potential to ± 100 mV. Representative tracings (control, followed by the whole-cell tracings while perfusing the test solution) were made on an average of $n=12$ cells. Composite 'delta' tracings were obtained by subtraction of the test average values, from those under control conditions. The whole-cell conductance obtained from the current -to-voltage relationships was highly linear under both conditions, and reflected 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 was near zero mV. Values were comparatively similar to those obtained with the zero mV protocol.

Applicants also determined whole-cell currents under basal conditions, with a protocol stepping from -120 mV holding potential to ± 100 mV. Representative tracings (control, followed by the whole-cell tracings while perfusing the test solution) were made on an average of $n=12$ cells. Composite 'delta' tracings were obtained by subtraction of the test average values, from those under control conditions. The whole-cell conductance obtained from the current -to-voltage relationships was highly linear under both conditions, and reflected 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 was near zero mV. Values were comparatively similar to those obtained with the zero mV protocol.

In addition, Applicants determined 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. Representative tracings (control, followed by the whole-cell tracings after cAMP stimulation, followed by perfusion with the drug-containing solution) were made on an average of $n=12$ cells. Composite 'delta' tracings (corresponding to voltage protocols at zero mV, and at -40 mV) were obtained by subtraction of the test average values in drug + cAMP, from those under control conditions (cAMP alone).

The whole-cell conductance obtained from the current-to-voltage relationships was highly linear under all conditions, and reflected a change in conductance due to the test conditions.

Applications demonstrated whole-cell currents under cAMP-stimulated conditions, obtained with protocols stepping from various holding potentials to ± 100 mV. Representative tracings (control, followed by the whole-cell tracings after cAMP stimulation, followed by perfusion with the drug-containing solution) were made on an average of $n=5$ cells. Composite 'delta' tracings (voltage protocols at -60 mV, and -120 mV) were obtained by subtraction of the test average values in drug + cAMP, from those under control conditions (cAMP alone). The whole-cell conductance, obtained from the current-to-voltage relationships, was highly linear under all conditions, and reflected a change in conductance due to the test conditions.

Applicants also demonstrated 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). 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 was further observed in the subtracted currents, 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.

5 Taken together with Applicants' other data, 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 signaling systems, comprising use of the inventive electrokinetically generated solutions to
10 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, and TSLP. 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.

15

EXAMPLE 7

(Effects of inventive electrokinetically-altered fluids on whole-cell conductance were determined)

Overview. Patch clamp analysis conducted on Calu-3 cells perfused with inventive
20 electrokinetically generated fluids (RNS-60 and Solas) revealed that (i) exposure to RNS-60 and Solas resulted in increases in whole cell conductance, (ii) that exposure of cells to the RNS-60 produced an increase in a non-linear conductance, evident at 15 min incubation times, and (iii) that exposure of cells to the RNS-60 produced an effect of RNS-60 saline on calcium permeable channels. Applicants performed patch clamp studies to further confirm the utilities, as described
25 herein, of the inventive electrokinetically generated saline fluids (RNS-60 and Solas), including the utility to modulate whole-cell currents. Two sets of experiments were conducted.

The summary of the data of the *first* set of experiments indicates that the whole cell conductance (current-to-voltage relationship) obtained with Solas saline is highly linear for both
30 incubation times (15 min, 2 hours), and for all voltage protocols. It is however evident, that longer incubation (2 hours) with Solas increased the whole cell conductance. Exposure of cells to the RNS-60 produced an increase in a non-linear conductance, as shown in the delta currents (Rev-Sol subtraction), which is only evident at 15 min incubation time. The effect of the RNS-60 on this non-linear current disappears, and is instead highly linear at the two-hour incubation

time. The contribution of the non-linear whole cell conductance, as previously observed, was voltage sensitive, although present at all voltage protocols.

The summary of data of the *second* set of experiments indicates that there is an effect of the RNS-60 saline on a non-linear current, which was made evident in high calcium in the external solution. The contribution of the non-linear whole cell conductance, although voltage sensitive, was present in both voltage protocols, and indicates an effect of RNS-60 saline on calcium permeable channels.

First set of experiments (increase of conductance and activation of a non-linear voltage regulated conductance)

Methods for first set of experiments. See above for general patch clamp methods. In the following first set of experiments, patch clamp studies were performed to further confirm the utility of the inventive electrokinetically generated saline fluids (RNS-60 and Solas) to modulate whole-cell currents, using Calu-3 cells under basal conditions, with protocols stepping from either zero mV holding potential, -120 mV, or -60 mV.

The whole-cell conductance in each case was obtained from the current-to-voltage relationships obtained from cells incubated for either 15 min or two hour. In this study, groups were obtained at a given time, for either Solas or RNS-60 saline solutions. The data obtained are expressed as the mean $\pm$ SEM whole cell current for 5-9 cells.

Results. Figures 3 A-C show the results of a series of patch clamping experiments that assessed the effects of the electrokinetically generated fluid (e.g., RNS-60 and Solas) on epithelial cell membrane polarity and ion channel activity at two time-points (15 min (left panels) and 2 hours (right panels)) and at different voltage protocols (A, stepping from zero mV; B, stepping from -60 mV; and C, stepping from -120 mV). The results indicate that the RNS-60 (filled circles) has a larger effect on whole-cell conductance than Solas (open circles). In the experiment similar results were seen in the three voltage protocols and at both the 15 minute and two-hour incubation time points.

Figures 4 A-C show graphs resulting from the subtraction of the Solas current data from the RNS-60 current data at three voltage protocols ("Delta currents") (A, stepping from zero mV; B, stepping from -60 mV; and C, stepping from -120 mV) and the two time-points (15 mins (open circles) and 2 hours (filled circles)). These data indicated that at the 15 minute time-point with RNS-60, there is a non-linear voltage-dependent component that is absent at the 2 hour time point.

As in previous experiments, data with "Normal" saline gave a very consistent and time-independent conductance used as a reference. The present results were obtained by matching

groups with either Solas or RNS-60 saline, and indicate that exposure of Calu-3 cells to the RNS-60 saline under basal conditions (without cAMP, or any other stimulation), produces time-dependent effect(s), consistent with the activation of a voltage-regulated conductance at shorter incubation times (15 min). This phenomenon was not as apparent at the two-hour incubation point. As described elsewhere herein, the linear component is more evident when the conductance is increased by stimulation with the cAMP “cocktail”. Nonetheless, the two-hour incubation time showed higher linear conductance for both the RNS-60 and the Solas saline, and in this case, the RNS-60 saline doubled the whole cell conductance as compared to Solas alone. This evidence indicates that at least two contributions to the whole cell conductance are affected by the RNS-60 saline, namely the activation of a non-linear voltage regulated conductance, and a linear conductance, which is more evident at longer incubation times.

Second set of experiments (effect on calcium permeable channels)

Methods for second set of experiments. See above for general patch clamp methods. In the following second set of experiments, yet additional patch clamp studies were performed to further confirm the utility of the inventive electrokinetically generated saline fluids (RNS-60 and Solas) to modulate whole-cell currents, using Calu-3 cells under basal conditions, with protocols stepping from either zero mV or -120 mV holding potentials.

The whole-cell conductance in each case was obtained from the current-to-voltage relationships obtained from cells incubated for 15 min with either saline. To determine whether there is a contribution of calcium permeable channels to the whole cell conductance, and whether this part of the whole cell conductance is affected by incubation with RNS-60 saline, cells were patched in normal saline after the incubation period (entails a high NaCl external solution, while the internal solution contains high KCl). The external saline was then replaced with a solution where NaCl was replaced by CsCl to determine whether there is a change in conductance by replacing the main external cation. Under these conditions, the same cell was then exposed to increasing concentrations of calcium, such that a calcium entry step is made more evident.

Results: Figures 5 A-D show the results of a series of patch clamping experiments that assessed the effects of the electrokinetically generated fluid (e.g., Solas (panels A and B) and RNS-60 (panels C and D)) on epithelial cell membrane polarity and ion channel activity using different external salt solutions and at different voltage protocols (panels A and C show stepping from zero mV, whereas panels B and D show stepping from -120 mV). In these experiments one time-point of 15 minutes was used. For Solas (panels A and B) the results indicate that: 1) using CsCl (square symbols) instead of NaCl as the external solution, increased whole cell

conductance with a linear behavior when compared to the control (diamond symbols); and 2) CaCl_2 at both 20 mM CaCl_2 (circle symbols) and 40 mM CaCl_2 (triangle symbols) increased whole cell conductance in a non-linear manner. For RNS-60 (panels C and D), the results indicate that: 1) using CsCl (square symbols) instead of NaCl as the external solution had little
5 effect on whole cell conductance when compared to the control (diamond symbols); and 2) CaCl_2 at 40 mM (triangle symbols) increased whole cell conductance in a non-linear manner.

Figures 6 A-D show the graphs resulting from the subtraction of the CsCl current data (shown in Figure 5) from the 20 mM CaCl_2 (diamond symbols) and 40 mM CaCl_2 (square symbols) current data at two voltage protocols (panels A and C, stepping from zero mV; and B and D, stepping from -120 mV) for Solas (panels A and B) and RNS-60 (panels C and D). The
10 results indicate that both Solas and RNS-60 solutions activated a calcium-induced non-linear whole cell conductance. The effect was greater with RNS-60 (indicating a dosage responsiveness), and with RNS-60 was only increased at higher calcium concentrations. Moreover, the non-linear calcium dependent conductance at higher calcium concentration was
15 also increased by the voltage protocol.

The data of this second set of experiments further indicates an effect of RNS-60 saline and Solas saline for whole cell conductance data obtained in Calu-3 cells. The data indicate that 15-min incubation with either saline produces a distinct effect on the whole cell conductance, which is most evident with RNS-60, and when external calcium is increased, and further
20 indicates that the RNS-60 saline increases a calcium-dependent non-linear component of the whole cell conductance.

The accumulated evidence suggests activation by Revalessio saline of ion channels, which make different contributions to the basal cell conductance.

Taken together with Applicants' other data (e.g., the data of Applicants other working
25 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, lipid components, or intracellular components with are exchangeable by the cell (e.g., signaling pathways, such as calcium dependant cellular signaling systems, comprising use of the
30 inventive electrokinetically generated solutions to impart electrochemical and/or conformational changes in membranous structures (e.g., membrane and/or membrane proteins, receptors or other membrane 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.

EXAMPLE 8

(Effects of inventive electrokinetically-altered fluids on whole-cell conductance were investigated, and a dose response curve was generated)

5 *Overview.* In this experiment Applicants assessed the effect of dilutions of the electrokinetically-altered fluid (e.g., RNS-60) on epithelial cell membrane polarity and ion channel activity.

10 *Methods.* See above for general patch clamp methods. In the following experiment, patch clamp studies were performed to further confirm the utility of the inventive electrokinetically generated saline fluids (RNS-60) to modulate whole-cell currents. In particular, the experiment assessed the effect of dilutions of the inventive electrokinetically generated saline fluid. The solutions were made by diluting the inventive electrokinetically generated saline fluid in normal saline at concentrations of: 100% (Rev), 75% (3:4), 50% (1:1), 25% (4:3), and 0% (Sal).

15 *Results.* Figures 7 A and B show the results of a series of patch clamp experiments that assessed the effects of diluted electrokinetically generated fluid (e.g., RNS-60) on epithelial cell membrane polarity and ion channel activity. Panel A demonstrates the volts versus current of whole cell conductance for each diluted sample as indicated on the graph (Rev, 3:4, 1:1, 4:3, and Sal). Panel B demonstrates the dilution amount versus the change in current comparing the dilution to normal saline. The results indicate that the mechanism of action of the RNS-60 solution occurs in a linear dose responsive manner.

20

EXAMPLE 9

25 *(Treatment of primary bronchial epithelial cells (BEC) with the inventive electrokinetically generated fluids, as well as with non-electrokinetic control pressure pot fluid, resulted in reduced expression and/or activity of two key proteins of the airway inflammatory pathways, MMP9 and TSLP)*

30 *Overview.* Applicants have now shown (using Bio-Layer Interferometry biosensor, Octet Rapid Extended Detection (RED) (forteBioTM)), that in the presence of electrokinetically generated fluids (e.g., Rev; gas-enriched electrokinetically generated fluid) of the instant disclosure compared to normal saline, Bradykinin binding to the B2 receptor was concentration dependent, and binding affinity was increased. Additionally, in the context of T-regulatory cells stimulated with diesel exhaust particulate matter (PM, standard commercial source), Applicants' 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), 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).

5 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, Diphtheria toxin (DT390, a truncated diphtheria toxin molecule; 1:50 dilution of std. commercial concentration) resulted in

10 beta blockade, GPCR blockade and Ca channel blockade of the effects the activity of the electrokinetically generated fluids on Treg and PBMC function. Furthermore, Applicants' has shown that upon exposure to the inventive fluids, tight junction related proteins (e.g., JAM 2 and 3, GJA1, 3, 4 and 5 (junctional adherens), OCLN (occludin), claudins (e.g., CLDN 3, 5, 7, 8, 9, 10), TJP1 (tight junction protein 1)) were upregulated in lung tissue. Furthermore, as shown in

15 patch clamp studies, 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

20 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

25 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, non-electrokinetic control pressure pot fluid, 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

30 analysis. Both MMP9 and TSLP receptor antibodies were obtained from BD Biosciences and used as per manufacturer's specifications.

Results:

In Figures 1 and 2, 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
5 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. "PP60" represents cells exposed to the non-electrokinetic control pressure pot fluid, and "DEP + PP60" refers to cells treated with particulate matter in the presence of the non-electrokinetic control pressure pot fluid (i.e., having
10 60 ppm dissolved oxygen).

Figure 1 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 DEP induced TSLP receptor expression, while Normal Saline failed to produce similar level of reduction in DEP induced TSLP receptor expression (approximately 20%
15 reduction). Additionally, the non-electrokinetic control pressure pot fluid PP60 resulted in approximately 50% reduction in DEP induced TSLP receptor expression.

The effect of the inventive Revera 60, Solas, and also of the PP60 solutions 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
20 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
25 print)).

Likewise, Figure 2 shows the effect of Revera 60, Solas, non-electrokinetic control pressure pot fluid (PP60), 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%,
30 whereas normal saline (NS) had a marginal effect of about 20% reduction. Additionally, the non-electrokinetic control pressure pot fluid PP60 resulted in approximately 30% reduction in DEP-induced cell surface attached MMP9 levels. 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 patients with 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.

According to yet additional aspects, non-electrokinetic control pressure pot fluid (i.e., having 60 ppm dissolved oxygen) have 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. Without being bound by mechanism, Applicants' collective data indicates that the action of the non-electrokinetic control pressure pot fluid in this system is mediated by a mechanism that is distinct from that of Applicants' electrokinetically-generated fluids. This is not only because the effects are relatively smaller, but also because non-electrokinetic control pressure pot fluid has not displayed activity in other assays displaying activity with Applicants' electrokinetically generated fluids. Nonetheless, Applicants' discovery of the herein disclosed activity of non-electrokinetic control pressure pot fluid in this system represents a novel use for such pressure pot fluid in the context of asthma and related conditions as disclosed herein.

According to particular aspects, therefore, the inventive methods comprising administration of Applicants' electrokinetically generated fluids provide for modulation (down-regulation of TSLP expression and/or activity) are applicable to the treatment of at least one

disease or condition selected from the TSLP-mediated group consisting of disorders of the immune system, allergic inflammation, allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, and food allergies, inflammatory arthritis, rheumatoid arthritis and psoriasis.

The results disclosed herein are entirely consistent with the art-recognized role of TSLP as a master switch of allergic inflammation at the epithelial cell-DC interface (Yong-Jun et al., *J. Exp. Med.*, 203:269-273, 2006), and are further consistent with the phenotypes of mice lacking the TSLPR (*e.g.*, fail to develop asthma in response to inhaled antigens; Zhou et al., *supra* and Al-Shami et al., *J. Exp. Med.*, 202:829-839, 2005), and with results obtained from pretreating OVA-DCs with anti-TSLPR (*e.g.*, resulting in a significant reduction of eosinophils and lymphocyte infiltration as well as IL-4 and IL-5 levels).

The presently disclosed subject matter further illuminates the role that TSLPR plays in DC-primed allergic disease, and provides for novel compositions and methods comprising administration of Applicants' electrokinetic ally generated fluids.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for treating a TSLP-mediated or TSLPR-mediated disease or condition, comprising administration to a mammal in need thereof, a therapeutically effective amount of an ionic aqueous solution of charge-stabilized oxygen-containing nanobubbles having an average diameter of less than 100 nanometers sufficient for treating a TSLP-mediated or TSLPR-mediated disease or condition, wherein the TSLP-mediated or TSLPR-mediated disease or condition of the immune system comprises at least one selected from the group consisting of allergic inflammation and inflammatory arthritis.
2. The method of claim 1, wherein the charge-stabilized oxygen-containing nanobubbles are stably configured in the ionic aqueous fluid in an amount sufficient to provide, upon contact of a living cell by the fluid, modulation of at least one of cellular membrane potential and cellular membrane conductivity.
3. The method of claim 1, wherein the charge-stabilized oxygen-containing nanobubbles are the major charge-stabilized gas-containing nanostructure species in the fluid.
4. The method of claim 1, wherein the percentage of dissolved oxygen molecules present in the fluid as the charge-stabilized oxygen-containing nanobubbles is a percentage selected from the group consisting of greater than: 0.01%, 0.1%, 1%, 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; and 95%.
5. The method of claim 1, wherein the total dissolved oxygen is substantially present in the charge-stabilized oxygen-containing nanobubbles.
6. The method of claim 1, wherein the charge-stabilized oxygen-containing nanobubbles have an average diameter of less than a size selected from the group consisting of: 90 nm; 80 nm; 70 nm; 60 nm; 50 nm; 40 nm; 30 nm; 20 nm; 10 nm; and less than 5 nm.
7. The method of claim 1, wherein the ionic aqueous solution comprises a saline solution.
8. The method of claim 1, wherein the fluid is superoxygenated.
9. The method of claim 1, wherein the fluid comprises a form of solvated electrons.
10. The method of any one of claims 1-9, wherein the disease or disorder of the immune system comprises allergic inflammation.

11. The method of claim 10, wherein the allergic inflammation comprises at least one of allergic airway inflammation, DC-mediated inflammatory Th2 responses, atopic dermatitis, atopic eczema, asthma, obstructive airways disease, chronic obstructive pulmonary disease, IgE-mediated disorders, rhino-conjunctivitis and food allergies.

12. The method of any one of claims 1-9, wherein the TSLP-mediated or TSLPR-mediated disease or condition comprises inflammatory arthritis.

13. The method of claim 12, wherein the inflammatory arthritis comprises at least one of rheumatoid arthritis and psoriasis.

14. The method of any one of claims 1-9, further comprising combination therapy, wherein at least one additional therapeutic agent is administered to the patient.

15. The method of claim 14, wherein the at least one additional therapeutic agent is selected from the group consisting of short-acting β_2 -agonists, long-acting β_2 -agonists, anticholinergics, corticosteroids, systemic corticosteroids, mast cell stabilizers, leukotriene modifiers, methylxanthines, and combinations thereof.

16. The method of claim 14, wherein the at least one additional therapeutic agent is selected from the group consisting of: bronchodilators consisting of β_2 -agonists including albuterol, levalbuterol, pirbuterol, arformoterol, formoterol, salmeterol, and anticholinergics such as ipratropium and tiotropium; corticosteroids including beclomethasone, budesonide, flunisolide, fluticasone, mometasone, triamcinolone, methyprednisolone, prednisolone, prednisone; leukotriene modifiers including montelukast, zafirlukast, and zileuton; mast cell stabilizers including cromolyn and nedocromil; methylxanthines including theophylline, combination drugs including ipratropium and albuterol, fluticasone and salmeterol, budesonide and formoterol; antihistamines including hydroxyzine, diphenhydramine, loratadine, cetirizine, and hydrocortisone; immune system modulating drugs including tacrolimus and pimecrolimus; cyclosporine; azathioprine; mycophenolatemofetil; and combinations thereof.

17. The method of claim 14, wherein the at least one additional therapeutic agent is a TSLP and/or TSLPR antagonist.

18. The method of claim 17, wherein the TSLP and/or TSLPR antagonist is selected from the group consisting of neutralizing antibodies specific for TSLP and the TSLP receptor, soluble TSLP receptor molecules, and TSLP receptor fusion proteins, including TSLPR-immunoglobulin Fc molecules or polypeptides that encode components of more than one receptor chain.

19. The method of any one of claims 1-9, comprising administration of the electrokinetic fluid to a cell network or layer, and further comprising modulation of an intercellular junction therein.

20. The method of claim 19, wherein the intracellular junction comprises at least one selected from the group consisting of tight junctions, gap junctions, zona adherens and desmosomes.

21. The method of claim 19, wherein the cell network or layers comprises at least one selected from the group consisting of pulmonary epithelium, bronchial epithelium, and intestinal epithelium.

22. The method of any one of claims 1-9, wherein the ionic aqueous fluid is oxygenated, and wherein the oxygen in the fluid is present in an amount selected from the group consisting 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.

23. The method of claim 2, wherein the ability to modulate at least one of cellular membrane potential and cellular membrane conductivity persists for a time period selected from the group consisting of at least two, at least three, at least four, at least five, at least 6, and at least 12 months, in a closed gas-tight container.

24. The method of any one of claims 1-9, wherein the amount of oxygen present in charge-stabilized oxygen-containing nanobubbles of the electrokinetically-altered fluid is selected from the group consisting of at least 8 ppm, at least 15, ppm, at least 20 ppm, at least 25 ppm, at least 30 ppm, at least 40 ppm, at least 50 ppm, and at least 60 ppm oxygen, at atmospheric pressure.

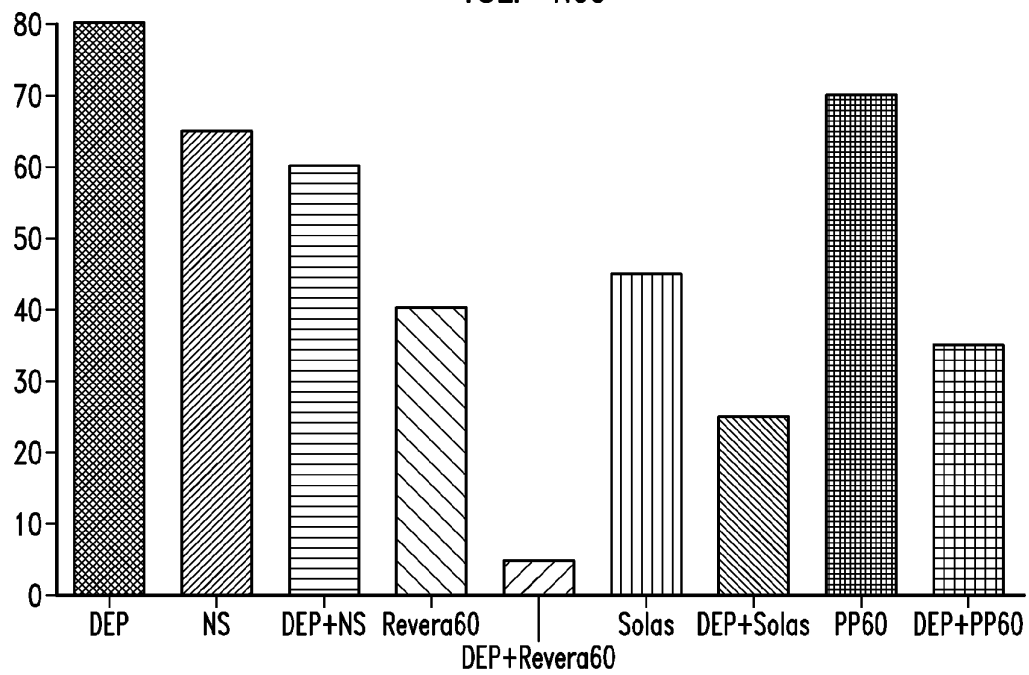
25. The method of any one of claims 1-9, wherein treating comprises administration by at least one of topical, inhalation, intranasal, and intravenous.

26. Use of a therapeutically effective amount of an ionic aqueous solution of charge-stabilized oxygen-containing nanobubbles having an average diameter of less than 100 nanometers sufficient for treating a TSLP-mediated or TSLPR-mediated disease or condition in preparation of a medicament for treatment of a TSLP-mediated or TSLPR-mediated disease or condition.

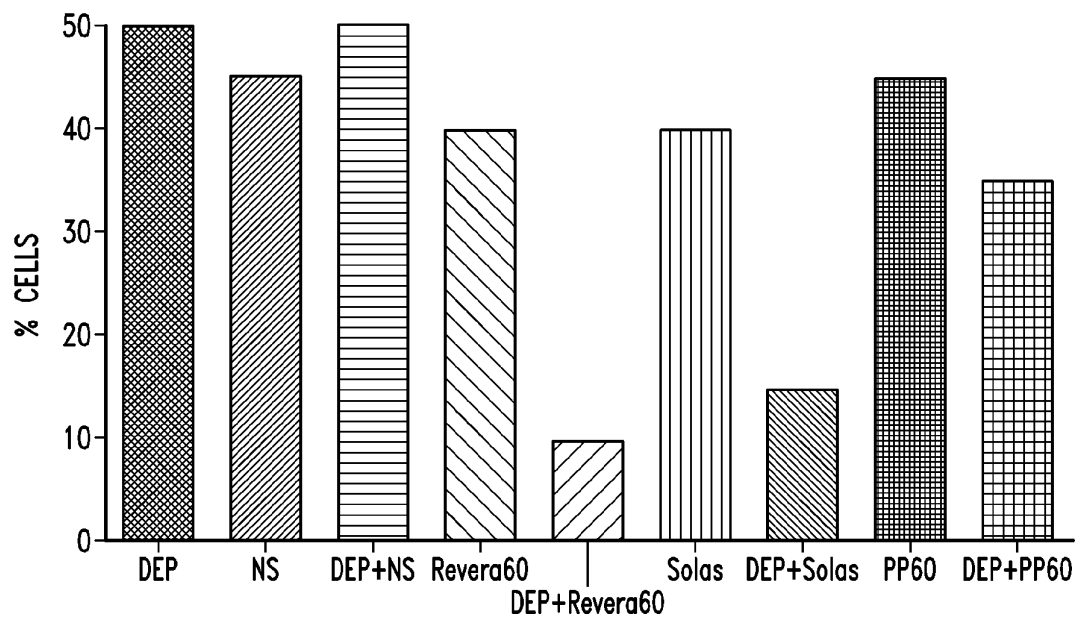
27. The method of claim 1, or the use of claim 26, substantially as hereinbefore described.

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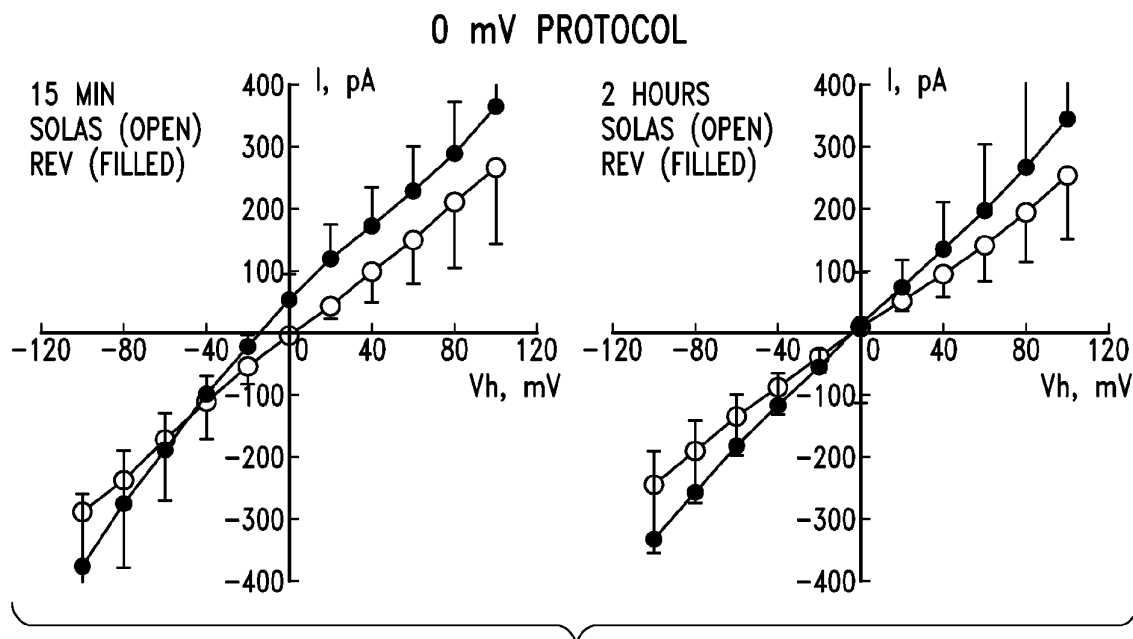
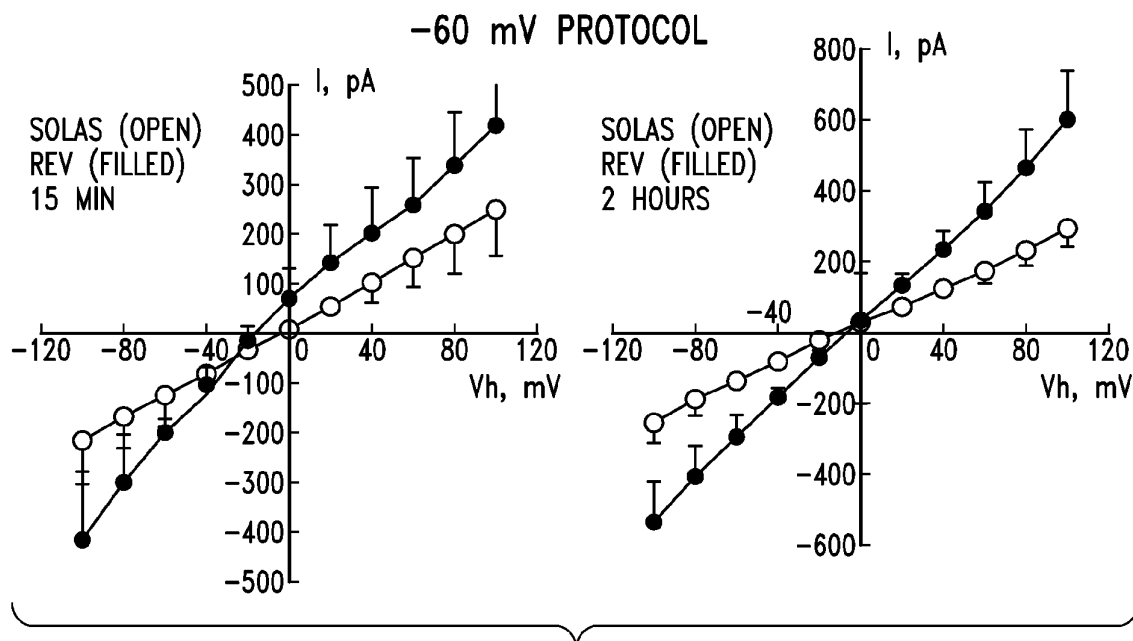
TSLP Rec

*Fig. 1*

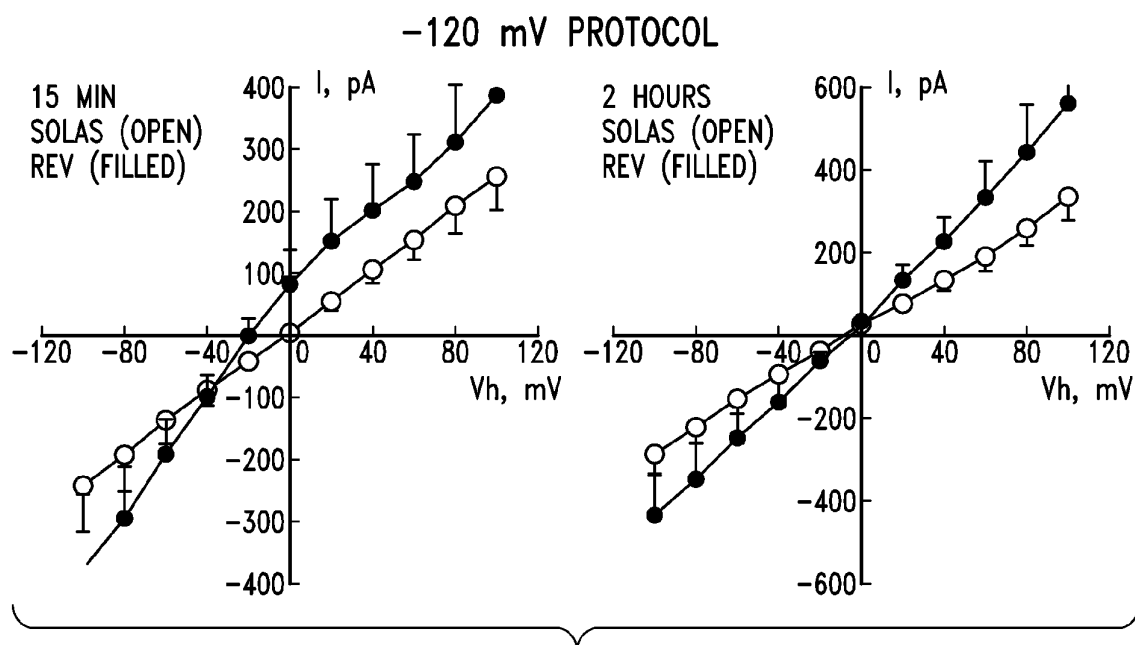
MMP9

*Fig. 2*

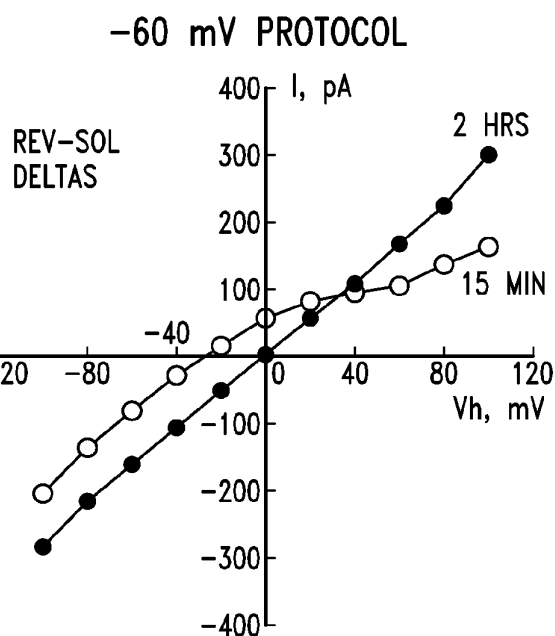
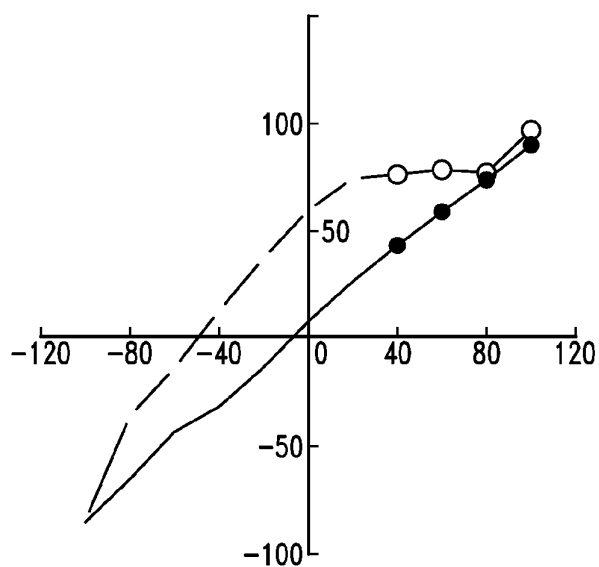
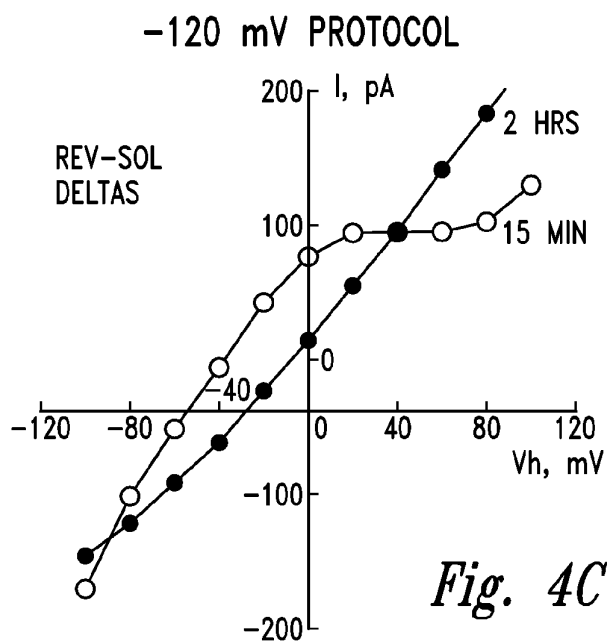
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*Fig. 3A**Fig. 3B*

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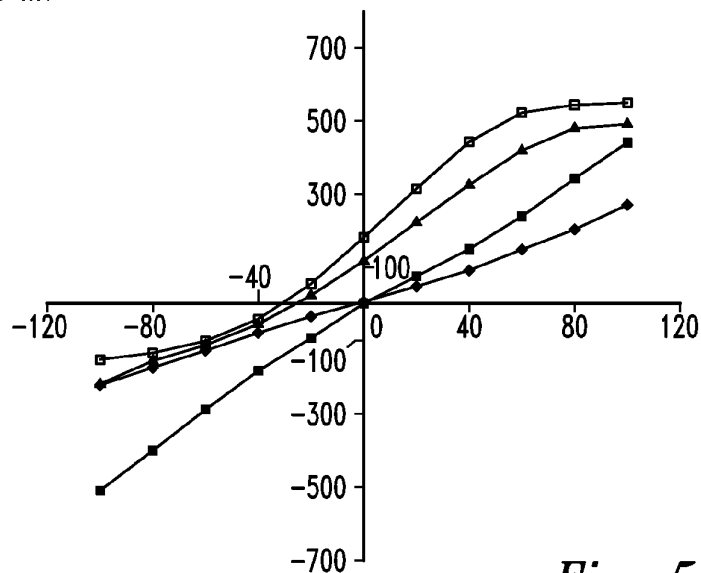
*Fig. 3C*

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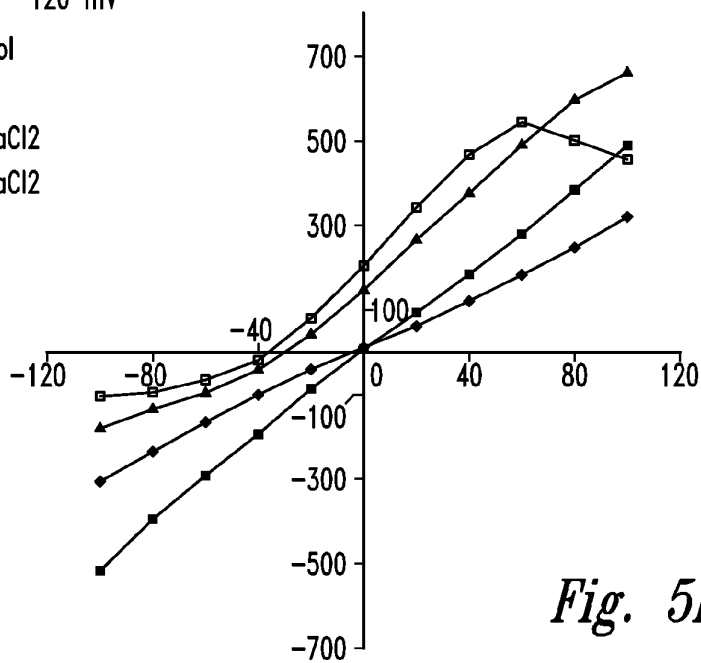
*Fig. 4B**Fig. 4C*

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Solus 15 min, 0 mV

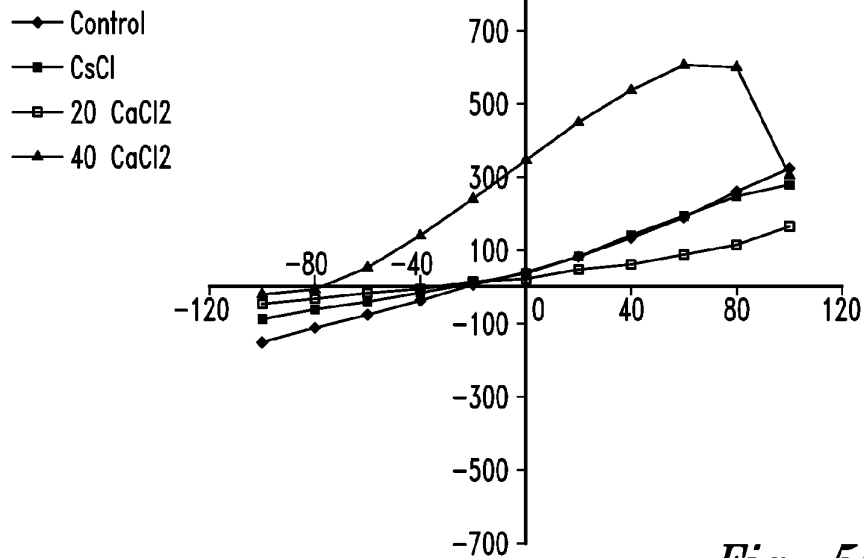
*Fig. 5A*

Solus 15 min, -120 mV

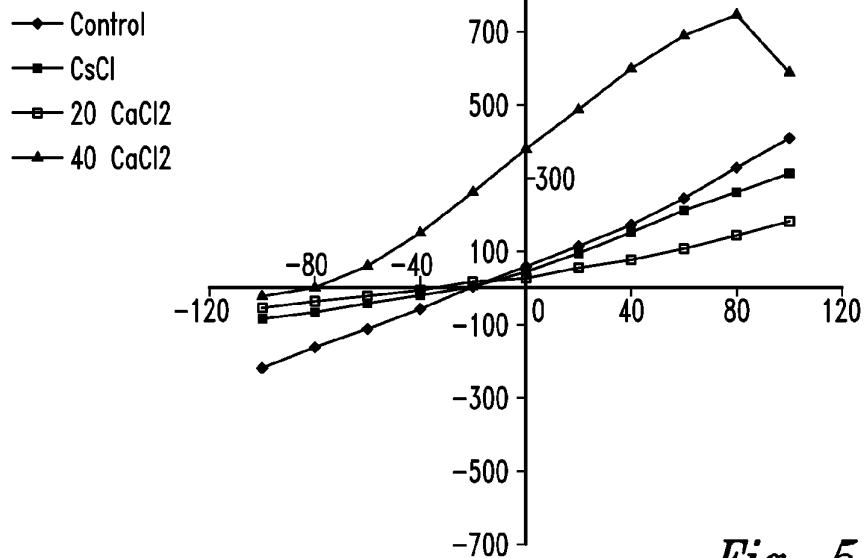
*Fig. 5B*

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Rev 15 min, 0 mV

*Fig. 5C*

Rev 15 min, -120 mV

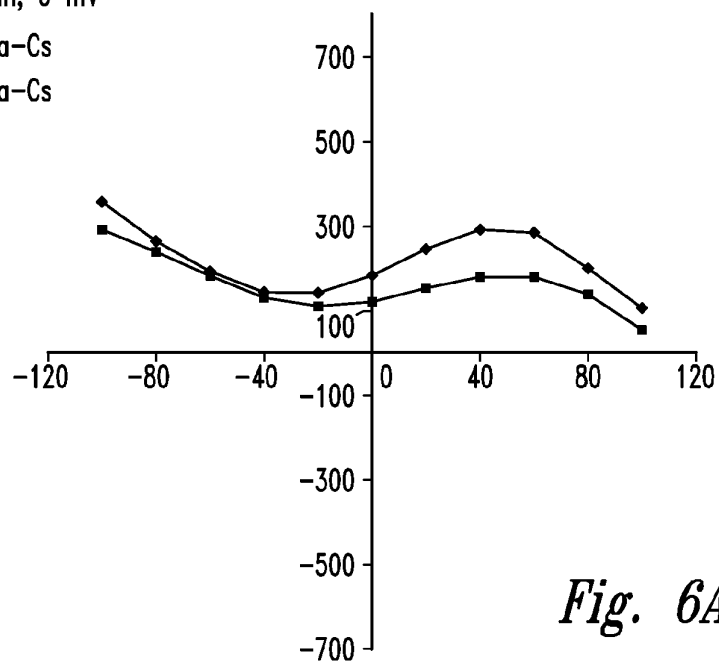
*Fig. 5D*

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Solas 15 min, 0 mV

—◆— 20 Ca-Cs

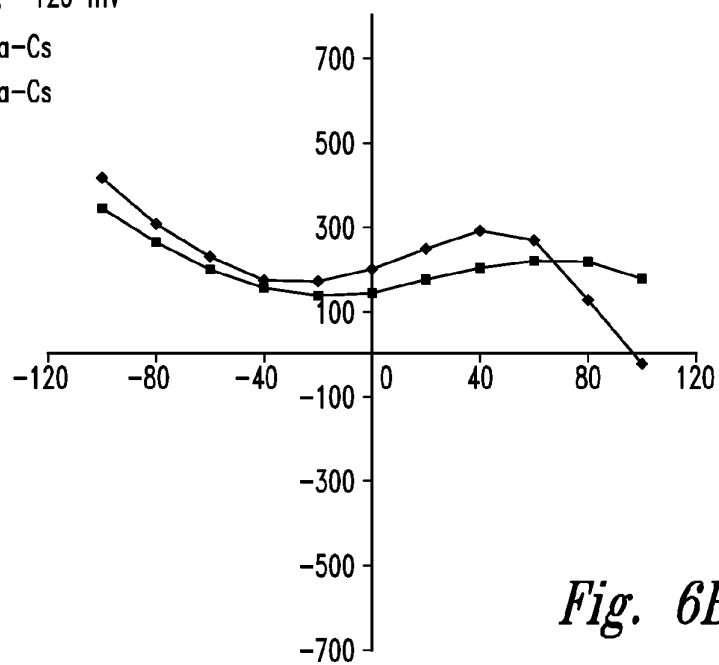
—■— 40 Ca-Cs

*Fig. 6A*

Solas 15 min, -120 mV

—◆— 20 Ca-Cs

—■— 40 Ca-Cs

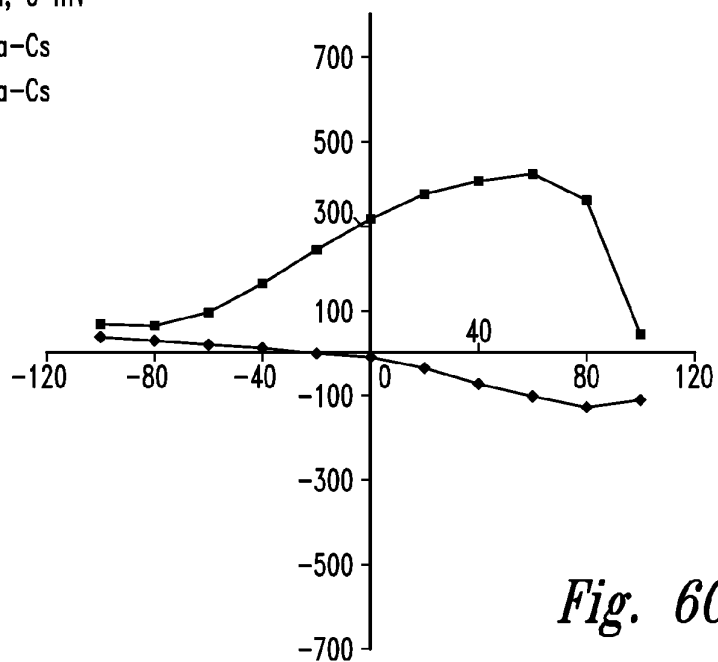
*Fig. 6B*

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Rev 15 min, 0 mV

—◆— 20 Ca-Cs

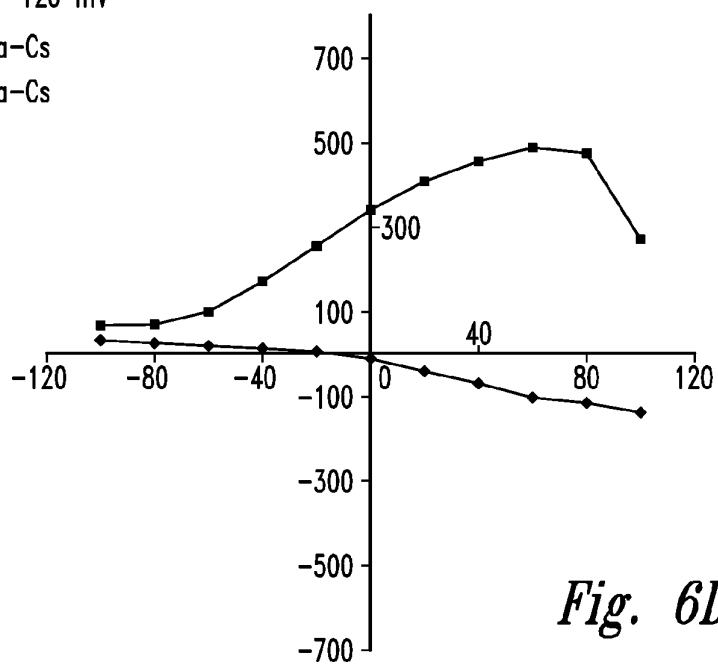
—■— 40 Ca-Cs

*Fig. 6C*

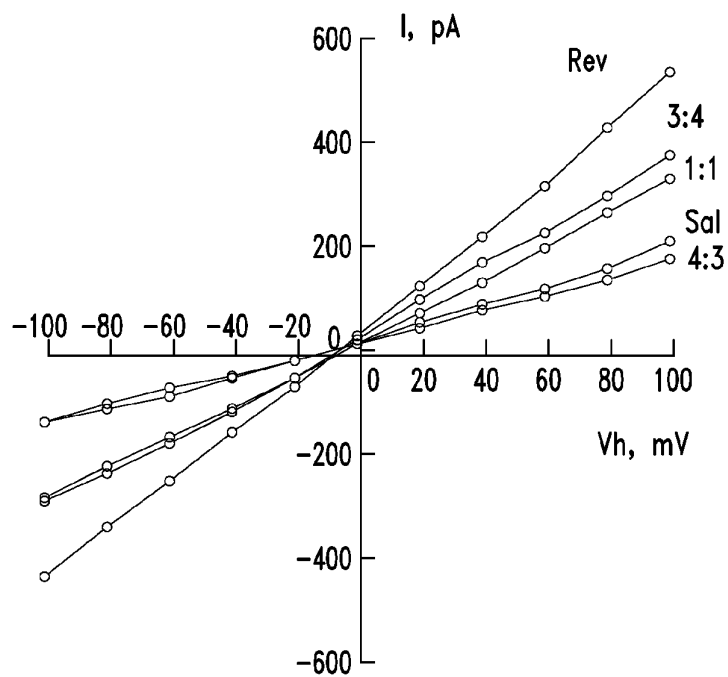
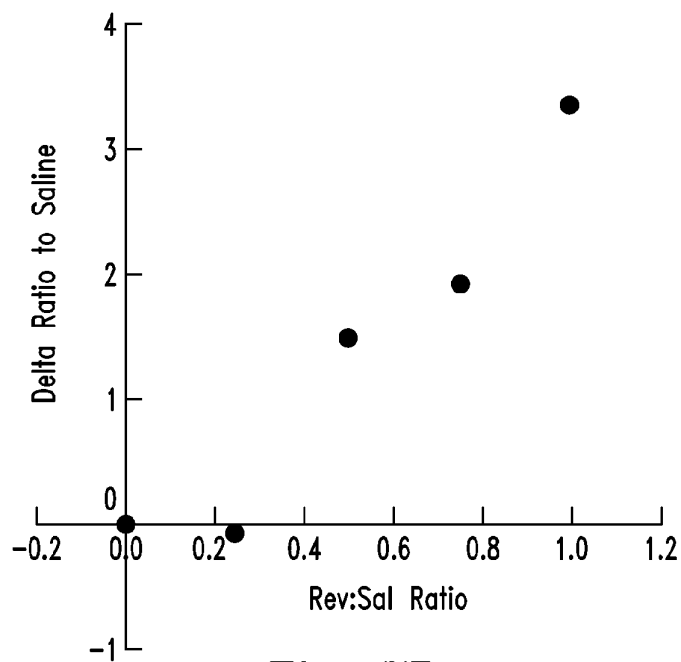
Rev 15 min, -120 mV

—◆— 20 Ca-Cs

—■— 40 Ca-Cs

*Fig. 6D*

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*Fig. 7A**Fig. 7B*