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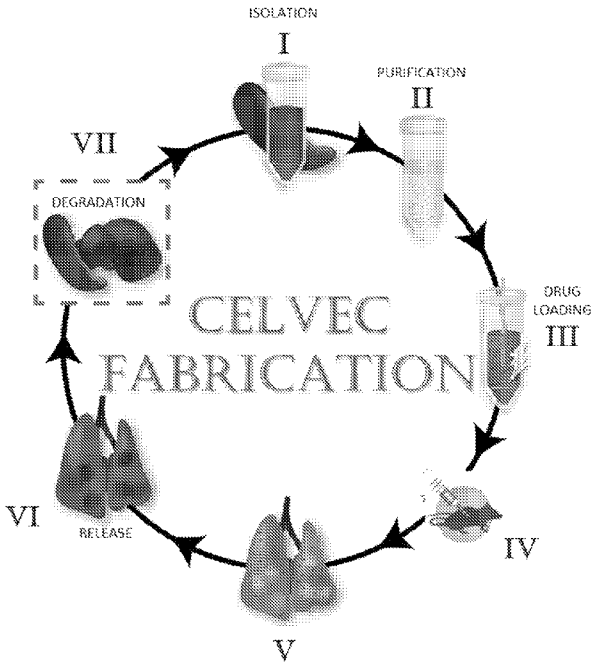

FIG. 1

(57) Abstract: Disclosed are compositions and methods for electroporation that provide for high payload of a therapeutic agent into a cell. Electroporation buffer solutions comprising a plurality of buffering agents, at least one salt, a carbon polyol, and optionally an acid, wherein the plurality of buffering agents and acid are in an amount such that the pH of the electroporation buffer solution is from about 4.5 to about 5 are disclosed. Kits comprising the electroporation buffer solutions, and optionally a therapeutic agent, are also disclosed. Methods of introducing doxorubicin into immune cells to form a cellular vector, using the electroporation buffer solutions, and compositions produced therefrom are also described. The compositions comprising the cellular vectors can be used to treat a cancerous tissue in a subject, such as the lungs.

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CELLULAR VECTORS FOR DELIVERY OF THERAPEUTICS

FIELD

The disclosed subject matter relates generally to cellular vectors, more particularly to compositions and methods for making and using the cellular vectors.

BACKGROUND

Metastatic spreading from a tumor's primary site to secondary organs is always associated with a significant reduction in the overall survival of a patient, sometimes representing a "point of no return" characterized by organ failure and inadequate treatment options. Many efforts have been dedicated in the past decade to optimize the transport of pharmaceuticals by using drug delivery systems designed to accumulate at the cancer lesion. Targeted delivery systems can increase the efficacy of standard chemotherapeutics and reduce the onset of adverse effects (Gao et al. *Journal of Controlled Release* 2012, 162:45-55; Parveen et al. *Nanomedicine: Nanotechnology, Biology and Medicine* 2012, 8:147-166; and Wang et al. *Annual Review of Medicine* 2012, 63:185-198). The ideal drug delivery system should be able to transport and protect the therapeutic payload in the vascular system, target the cancer tissue, and maximize the release of the therapeutic payload only to cancer cells.

Targeting is usually based on tumor features that assume the existence of structurally well-defined cancer lesions. For example nanotherapeutics are designed to accumulate in the tumor microenvironment by exploiting defects in tumor neovasculature (Prabhakar et al. *Cancer research* 2013, 73:2412-2417). In particular, most of the nanodelivery platforms developed so far exploit the enhanced extravasation and prolonged retention effect (EPR) that characterizes cancer tissue allowing in some cases their clinical translation (Prabhakar et al.; Stylianopoulos T, *Therapeutic delivery* 2013, 4:421-423; and Torchilin V, *Advanced drug delivery reviews* 2011, 63:131-135). However, the EPR is not homogenously present in the tumor vasculature. Current clinical therapies aim to treat not only the cancer lesion, but also the surrounding tissues in order to guarantee the killing of cancer cells spread from the main cancer lesion. For example, surgical resection of primary and metastatic cancer is always accompanied by the removal of large areas of surrounding tissues (Bill-Axelson et al. *New England Journal of Medicine* 2011, 364:1708-1717; Guzzo et al., *Critical reviews in oncology/hematology* 2010, 74:134-148; and Veronesi et al., *New England Journal of Medicine* 2002, 347:1227-1232). After surgery the patients are treated with chemotherapy to decrease the probability of relapse of any residual tumor cells

(adjuvant therapy) (Ando et al., *Journal of Clinical Oncology* 2003, 21:4592-4596; Des Guetz et al., *European Journal of Cancer* 2010, 46:1049-1055; Sasako et al., *Journal of Clinical Oncology* 2011, 29, 4387-4393; and Voduc et al., *Journal of Clinical Oncology* 2010, 28:1684-1691). In addition, the translation of drug delivery systems based on synthetic approach is hampered by high efficiency of the immune system in recognizing and clearing foreign materials (Brown, S, *Nature Nanotechnology* 2013); toxicity of the nanocarriers and their degradation products (Eifler et al., *In Biomedical Nanotechnology*, Springer: 2011, pp 325-338; and Love et al., *Annual Review of Analytical Chemistry* 2012, 5:181-205); increasing costs of production (Bosetti et al., *Nanomedicine* 2011, 6:747-755 and Park, K., *ACS Nano* 2013, 7:7442-7447).

What are needed are compositions and methods to target the whole organ affected by the tumor, which can provide a significant advantage over traditional chemotherapy. Further, what are needed are compositions that exhibit fast clearance from the blood and/or exhibit low uptake in non-targeted tissues. Still further, what are needed are compositions that have high payload and high loading efficiency for therapeutics. The compositions and methods disclosed herein address these and other needs.

SUMMARY

Disclosed herein are compositions and methods for electroporation that provide for high payload of a therapeutic agent into a cell. In some aspects, methods of introducing doxorubicin, or another therapeutic agent, into nucleated cells to form a cellular vector using an electroporation buffer solution are described. The method can include (a) providing a mixture comprising doxorubicin or other therapeutic agent in an electroporation buffer having a pH of from about 4.5 to about 5, (b) contacting the mixture with the nucleated cells, (c) applying at least one voltage pulse between a plurality of opposing pairs of needle electrodes disposed in the mixture so as to establish an electric field in the nucleated cells sufficient to transfect doxorubicin or other therapeutic agent into the nucleated cells to form the cellular vectors, and (d) washing the cellular vectors with a wash buffer having a pH of from about 5 to about 8, wherein substantially all of the nucleated cells are non-viable after step (d). In some examples, the nucleated cells can comprise immune cells, for example, T-cells or leukocytes.

The concentration of doxorubicin or other therapeutic agent in the mixture can be from about 15 mg/mL to about 50 mg/mL, for example, about 40 mg/mL. The concentration of the nucleated cells in the mixture can be from about 1 x 10^5 cells/mL to about 1 x 10^8 cells/mL, for example, from about 7.5 x 10^5 cells/mL to about 40 x 10^6

cells/mL.

The voltage pulse applied to the mixture can be for a period of from about 1 millisecond to about 90 milliseconds.

The cellular vectors can comprise doxorubicin in an amount of from about 5 to about 400 pg/cellular vector such as from about 50 to about 400 pg/cellular vector or about 50 to about 200 pg/cellular vector. Other therapeutic agents, if used, can be present in the cellular vectors in the same amounts.

The cellular vectors can be washed 2 to 4 times with the wash buffer. In certain embodiments, the cellular vectors can be washed with sequentially increasing pH of the wash buffer, wherein the wash buffer has a pH of from about 6 to about 8.

The electroporation buffer solution can comprise (a) a zwitterionic buffering agent at a concentration of from about 1 mM to about 5 mM, (b) a phosphate buffering agent at a concentration of from about 5 mM to about 30 mM, (c) at least one salt, in an effective amount such that the ionic strength of the solution is about physiological, (d) a carbon polyol at a concentration of from about 200 mM to about 300 mM, and (e) optionally an acid.

In some example, the zwitterionic buffering agent in the disclosed electroporation buffer solution can be selected from 2-(N-morpholino)ethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3-(N-morpholino)propanesulfonic acid, 3-morpholino-2-hydroxypropanesulfonic acid, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid, piperazine-N,N'-bis(2-ethanesulfonic acid), N-(2-hydroxyethyl)piperazine-N'-(2- hydroxypropanesulfonic acid); N-(2-hydroxyethyl)piperazine-N'-(4- butanesulfonic acid), N-cyclohexyl-2-aminoethanesulfonic acid, N-cyclohexyl-3-aminopropanesulfonic acid, 4-(cyclohexylamino)-1-butanesulfonic acid, 3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid, and combinations thereof. For example, the zwitterionic buffering agent can include 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

The concentration of the phosphate buffering agent can be about 15 mM. The concentration of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid can be about 2 mM.

The carbon polyol can be selected from mannitol, sorbitol, sucrose, glycerol, polyethylene glycol, xylitol, arabitol, maltitol, erythritol, isomalt, lactitol, and combinations thereof. The concentration of the carbon polyol in the electroporation buffer solution can be about 250 mM. In some examples, the carbon polyol can be mannitol.

The disclosed electroporation buffer solutions can also include a surfactant. Suitable

surfactants include polysorbate surfactants.

Compositions comprising the cellular vectors prepared using the methods described herein are also disclosed. The compositions can be used to treat a cancerous tissue in the lungs of a subject. The method of treating the cancerous tissue in the subject can include administering to the subject a pharmaceutically effective amount of the composition. The composition can be administered intravenously. In some examples, the composition can be administered in an effective amount of from about 5 to about 10 mg/kg body weight of doxorubicin or other therapeutic agent.

Kits comprising the disclosed electroporation buffer solutions are also disclosed. The kit can include the disclosed electroporation buffer solution and a therapeutic agent. In some aspects, the therapeutic agent can be a chemotherapeutic agent such as doxorubicin or daunomycin. In some examples, the electroporation buffer solution and the therapeutic agent are provided in separate containers.

Additional advantages will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by practice of the aspects described below. The advantages described below will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive.

BRIEF DESCRIPTION OF THE FIGURES

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects described below.

Figure 1 is a schematic diagram showing the production of cellular vectors (CELVEC). Leukocytes may be obtained from several sources including intraperitoneal lavage, spleen, and blood (I). Specific cell phenotypes can be purified using a commercial kit (II), the loading can be performed through electroporation (III), the tumor bearing mice are injected with CELVEC (IV), the CELVEC reside in the lung for several hours (V) before being digested in liver and spleen (VI). After CELVEC injection, the cells are entrapped in the lung vasculature where they reside for about 4 hours: after this time, about 50% of CELVEC are cleared in liver and spleen, while after 24 hours, all the CELVEC are found in the organs.

Figures 2A-2H show the size of the CELVEC and the DOX payload accommodated within the cell cytoplasm. In Figures 2A and 2B are shown SEM pictures of a viable macrophages and CELVEC. After electroporation the cell shrinks in size and the surface of

CELVEC is completely affected losing their typical "rose" shape; in Figures 2C and 2D are shown fluorescent microscope images of a viable macrophage and CELVEC. After electroporation the cytoplasm of the cells is saturated with DOX. In Figures 2E and 2F are shown TEM images of a non-viable macrophage and CELVEC. After electroporation the cytoplasm ultrastructure is lost and it can be easily noted the nuclei of precipitated DOX. In Figures 2G and 2H are shown CELVEC at 2 different magnifications taken by an optical microscope.

Figures 3A-3C show loading and release in a typical electroporation experiment and the ability of the CELVEC to induce cell death towards 4T1 breast cancer cells. In Figure 3A is shown loading of DOX starting from a solution of 20 mgs/ml of DOX in a typical electroporation buffer. In Figure 3B is shown a typical passive release of the DOX from the CELVEC. More than the 80% of the drug is release in 8 hours. In Figure 3C is shown the ability of the CELVEC to kill 4T1 breast cancer cells. Compared to untreated control (st at 100%) and free DOX, CELVEC allowed for an enhanced cytotoxic effect of the therapeutic.

Figures 4A and 4B show biodistribution of CELVEC injected in healthy mice at early (Figure 4A) and late (Figure 4B) time points. CELVEC can efficiently target the lung and overtime is cleared in liver and spleen.

Figure 5 shows differential accumulation of DOX in different organs after encapsulation in CELVEC or administered as free therapeutic.

Figure 6 shows pulmonary accumulation of DOX encapsulated in CELVEC or administered as a free therapeutic.

DETAILED DESCRIPTION

Disclosed herein are compositions and methods for electroporation that provide for high payload of a therapeutic agent into a cell. The compositions and methods described herein may be understood more readily by reference to the following detailed description of specific aspects of the disclosed subject matter and the Examples included therein and to the Figures.

Before the present compositions and methods are disclosed and described, it is to be understood that the aspects described below are not limited to specific synthetic methods or specific reagents, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only and, unless a particular term is specifically defined herein, is not intended to be limiting.

Also, throughout this specification, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into

this application in order to more fully describe the state of the art to which the disclosed matter pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

Definitions

Throughout the description and claims of this specification the word "comprise" and other forms of the word, such as "comprising" and "comprises," means including but not limited to, and is not intended to exclude, for example, other additives, components, integers, or steps.

As used in the description and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a surfactant" includes mixtures of two or more such surfactants, reference to "an immune cell" includes mixtures of two or more such immune cells, reference to "the composition" includes mixtures of two or more of such compositions, and the like.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another aspect includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed.

The terms "treatment," "treat," "treating," and grammatical variations thereof, are used interchangeably herein to refer to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to

minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

The term "drug," as used herein, is used alternately with the term "therapeutic agent", and refers to a compound that is desirable to use in the body of an animal subject for a therapeutic and/or diagnostic purpose. Accordingly, the term "drug" encompasses, but is not limited to (i) conventional pharmaceutical compounds useful for the treatment of diseases or disorders, including, but not limited to, chemotherapeutic agents, anti-inflammatory agents, ionotropic agents, antimicrobial agents, etc.; and (ii) imaging agents such as detectable labels including but not limited to radioactive labels; paramagnetic labels, etc. The "drug," when administered to a subject, has a therapeutic and/or diagnostic effect and/or elicits a desired biological and/or pharmacological effect. In certain embodiments, the term "drug" as used herein refers to doxorubicin.

The terms "transfection," "transformation," and "introduction," and grammatical variations thereof, are used interchangeably herein to refer to the insertion of an exogenous molecule (e.g., a therapeutic agent), into a host cell, irrespective of the method used for the insertion, the molecular form of the molecule that is inserted, or the nature of the cell (e.g., prokaryotic or eukaryotic).

The term "subject" refers to any individual who is the target of administration or treatment. The subject can be a vertebrate, for example, a mammal. Thus, the subject can be a human or veterinary patient. The term "patient" refers to a subject under the treatment of a clinician, e.g., physician.

The term "therapeutically effective" refers to the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

Reference will now be made in detail to specific aspects of the disclosed materials, compounds, compositions, articles, and methods, examples of which are illustrated in the accompanying Examples.

Compositions

Electroporation buffer solutions having a pH of from about 4.5 to about 5 comprising a plurality of buffering agents, at least one salt, a carbon polyol, and optionally an acid are described herein. In some aspects, the electroporation buffer solution can

contain two or more buffering agents. In some further aspects, the plurality of buffering agents can include a zwitterionic buffering agent and at least one additional buffering agent.

Suitable examples of zwitterionic buffering agents include, but are not limited to, 2-(*N*-morpholino)ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO), 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid (EPPS or HEPPS), piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), *N*-(2-hydroxyethyl)piperazine-*N*'-(2- hydroxypropanesulfonic acid) (HEPPSO); *N*-(2-hydroxyethyl)piperazine-*N*'-(4- butanesulfonic acid) (HEPBS), *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES), *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), 4-(cyclohexylamino)-1-butanesulfonic acid (CABS), 3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid (CAPSO), and combinations thereof. In some examples, the zwitterionic buffering agent can be 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Suitable examples of additional buffering agents include, but are not limited to, phosphate buffering agents. Representative examples of phosphate buffering agents include potassium phosphate dibasic, sodium phosphate dibasic, potassium phosphate monobasic, sodium phosphate monobasic, and combinations thereof.

In some examples, the zwitterionic buffering agent can be in an amount such that the concentration is from about 0.5 mM to about 5 mM, such as from about 1 mM to about 5 mM, about 1 mM to about 4 mM, or about 1 mM to about 3 mM. In some examples, the zwitterionic buffering agent can be in an amount such that the concentration is about 0.5 mM, about 1 mM, about 1.5 mM, about 2 mM, about 2.5 mM, about 3 mM, about 3.5 mM, or about 4 mM.

In some examples, the additional buffering agent can be in an amount such that the concentration is from about 5 mM to about 50 mM, such as from about 5 mM to about 30 mM, about 10 mM to about 25 mM, or about 10 mM to about 20 mM. In some examples, the additional buffering agent can be in an amount such that the concentration is about 5 mM, about 7.5 mM, about 10 mM, about 12 mM, about 13 mM, about 14 mM, about 15 mM, or 16 mM.

The electroporation buffer solution can optionally include an acid or base, as required, such that the pH of the electroporation buffer solution is from about 4.5 to about 5. Suitable acid or base to adjust the pH of the electroporation buffer solution include, but are not limited to, hydrochloric acid, and sodium hydroxide. In some examples, the electroporation buffer solution can optionally include hydrochloric acid.

The electroporation buffer solution can include at least one salt, in an effective amount such that the ionic strength of the buffer solution is about physiological. Physiological ionic strength can be from about 100 mM to about 200 mM. In some examples, the at least one salt can be in an effective amount such that the ionic strength of the electroporation buffer solution is from about 100 mM to about 200 mM, for example, from about 100 mM to about 150 mM. In some examples, the at least one salt can be in an effective amount such that the ionic strength of the electroporation buffer solution is about 100 mM. Suitable salts that can be present in the electroporation buffer solution can include, but are not limited to, potassium chloride and sodium chloride.

The electroporation buffer solution can include a carbon polyol. In some examples, the carbon polyol can be an osmolyte. Carbon polyols, as used herein, refers to an alcohol containing multiple hydroxyl groups. In some embodiment, the carbon polyol is a 5 or 6 carbon polyol. In some examples, the carbon polyol can include one or more of mannitol, sorbitol, sucrose, glycerol, polyethylene glycol, xylitol, arabitol, maltitol, erythritol, isomalt, lactitol, or combinations thereof.

The carbon polyol can be present in the electroporation buffer at a concentration of from about 1 mM to about 500 mM. For example, the carbon polyol can be at a concentration of from about 150 mM to about 350 mM, or from about 200 to about 300 mM. In some examples, the carbon polyol, such as mannitol, can be present in the buffer at a concentration of about 250 mM.

In some examples, electroporation buffer solution described herein can have a pH of from about 4.5 to about 5 and contain (a) a zwitterionic buffering agent at a concentration of from about 1 mM to about 5 mM (e.g., about 2 mM), including HEPES, MES, MOPS, or combinations thereof, (b) a phosphate buffering agent at a concentration of from about 5 mM to about 50 mM (e.g., about 15 mM), including sodium phosphate dibasic, potassium phosphate dibasic, sodium phosphate monobasic, potassium phosphate monobasic, or combinations thereof, (c) at least one salt, in an effective amount such that the ionic strength of the solution is about physiological (e.g., about 100 mM), including potassium chloride and sodium chloride, (d) a carbon polyol at a concentration of from about 150 mM to about 350 mM (e.g., about 250 mM), including one or more of mannitol, sorbitol, sucrose, glycerol, polyethylene glycol, xylitol, arabitol, maltitol, erythritol, isomalt, and lactitol, and (e) optionally an acid such as hydrochloric acid.

In some examples, the electroporation buffer solution can include HEPES at a concentration of about 2 mM and a phosphate buffering agent at a concentration of from

about 15 mM.

The electroporation buffer solution can further comprise a surfactant. Suitable surfactants can be anionic, cationic, amphoteric or nonionic surface active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate, sulfonate and sulfate ions. Examples of anionic surfactants include sodium, potassium, ammonium of long chain alkyl sulfonates and alkyl aryl sulfonates such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium dodecylbenzene sulfonate; dialkyl sodium sulfosuccinates, such as sodium bis-(2-ethylthioxyl)-sulfosuccinate; and alkyl sulfates such as sodium lauryl sulfate. Cationic surfactants include, but are not limited to, quaternary ammonium compounds such as benzalkonium chloride, benzethonium chloride, cetrimonium bromide, stearyl dimethylbenzyl ammonium chloride, polyoxyethylene and coconut amine. Examples of nonionic surfactants include ethylene glycol monostearate, propylene glycol myristate, glyceryl monostearate, glyceryl stearate, polyglyceryl-4-oleate, sorbitan acylate, sucrose acylate, PEG-150 laurate, PEG-400 monolaurate, polyoxyethylene monolaurate, polysorbates, polyoxyethylene octylphenylether, PEG-1000 cetyl ether, polyoxyethylene tridecyl ether, polypropylene glycol butyl ether, PoloxamerTM 401, stearoyl monoisopropanolamide, and polyoxyethylene hydrogenated tallow amide. Examples of amphoteric surfactants include sodium N-dodecyl-beta-alanine, sodium N-lauryl-beta-iminodipropionate, myristoamphoacetate, lauryl betaine and lauryl sulfobetaine. In some examples, the electroporation buffer solution can include polysorbate 80, Tween 20, Tween 80, and combinations thereof.

Kits comprising the electroporation buffer solutions described herein are also disclosed herein. In some aspects, the kit comprises a therapeutic agent in addition to the electroporation buffer solution. The therapeutic agent can be a chemotherapeutic agent. In some examples, the kit comprises a chemotherapeutic agent selected from doxorubicin.

The kit can comprise one or more containers. For example, a kit can comprise an electroporation buffer solution and a chemotherapeutic agent provided separately from the buffer for addition thereto. In some examples, the chemotherapeutic agent can be provided as a powder in one container and the electroporation buffer solution is provided in a separate container.

The chemotherapeutic agent provided can be sufficient for a final concentration in the electroporation buffer solution of any concentration from about 15 mg/mL to about 100 mg/mL or from about 15 mg/mL to about 50 mg/mL. For example, the chemotherapeutic

agent, doxorubicin can be provided in an amount sufficient for a final concentration in the electroporation buffer solution of any concentration from about 15 mg/mL or greater, about 20 mg/mL or greater, about 25 mg/mL or greater, about 30 mg/mL or greater, about 35 mg/mL or greater, or about 50 mg/mL or less, about 45 mg/mL or less, about 40 mg/mL or less, about 35 mg/mL or less, or about 30 mg/mL or less.

Provided herein are also methods of making the electroporation buffer solutions described herein. The method can include mixing the plurality of buffering agents, at least one salt, and a carbon polyol. The pH of the final solution can be adjusted to from about 4.5 to about 5 by addition of an acid or base.

Methods of Use

Provided herein are also methods of using the electroporation buffer solutions described herein. For example, methods of introducing a therapeutic agent into nucleated cells to form cellular vectors using the electroporation buffer solution are described. The electroporation buffer solutions described herein can provide for improved electroporation across a wide variety of cell lines.

In some aspects, the nucleated cells used can be any suitable eukaryotic cell such as a bacterial cell, a yeast, plant or animal cell. In some examples, the nucleated cells can be a mammalian cell. In some examples, the nucleated cells are of human origin. Examples of suitable mammalian cells can include immune cells. Suitable immune cells include cells that recognize antigens from disease causing system. In some examples, the nucleated cells can be a T-cell, such as CD8 T cells, CD4 T cells, natural killer cells, natural killer T cells, stem cells, and leukocytes including macrophages. The nucleated cells can be derived from a healthy volunteer, a patient with cancer, or other form of tumor disease. In some examples, the nucleated cells are used for either autologous or allogeneic application.

The methods described herein can be used for introducing doxorubicin into nucleated cells. The method can include (a) providing a mixture comprising doxorubicin in an electroporation buffer solution described herein, (b) contacting the mixture with the nucleated cells, and (c) applying at least one voltage pulse between a plurality of opposing pairs of needle electrodes disposed in the mixture so as to establish an electric field in the nucleated cells sufficient to transfect doxorubicin into the nucleated cells to form the cellular vectors. The method can include (d) washing the cellular vectors with a wash buffer having a pH of from about 6 to about 8. Without wishing to be bound by theory, the method described is optimized to accommodate doxorubicin within the cytoplasm of the nucleated cells by exploiting the differences in solubility that doxorubicin shows in aqueous buffers as

a function of varying pH. In particular, step (d) washing the cellular vectors with a wash buffer allows for precipitation of doxorubicin encapsulated in the cellular vectors. The same protocol can be optimized for other therapeutic agents that exhibit differences in solubility as a function of pH.

The mixture provided in step (a) can include doxorubicin dissolved in the electroporation buffer solution using any suitable means, such as by sonication. In some embodiments, doxorubicin can be present in the mixture at a concentration of from about 15 mg/mL to about 50 mg/mL, such as from about 15 mg/mL to about 35 mg/mL or from about 15 mg/mL to about 25 mg/mL. In some embodiments, the nucleated cells can be present in the mixture at a concentration of from about 1 x 10^5 cells/mL to about 1 x 10^8 cells/mL. For example, the nucleated cells can be present at a concentration of from about 1 x 10^5 cells/mL to about 1 x 10^7 cells/mL, from about 1 x 10^5 cells/mL to about 1 x 10^6 cells/mL, or from about 7.5 x 10^5 cells/mL to about 40 x 10^6 cells/mL. The ratio of doxorubicin in the electroporation solution to nucleated cells can be high. In some examples, the weight ratio of doxorubicin to nucleated cells in the electroporation solution can be from 10^3:1 to 10^6:1 doxorubicin:cell.

The methods described herein can be practiced using standard electroporation protocols and any suitable electroporation devices, such as those manufactured by 4D-NUCLEOFECTOR™ Electroporating System (LONZA), BioRad (e.g., Gene Pulser MXcell System), and Sonidel, Ltd. In some examples, at least one voltage pulse is applied for a period of 90 milliseconds or less, 75 milliseconds or less, 60 milliseconds or less, 30 milliseconds or less, 20 milliseconds or less, 10 milliseconds or less, 5 milliseconds or less, or 1 millisecond or less.

Compositions comprising the cellular vectors (including the nucleated cells loaded with a therapeutic agent, for example, doxorubicin) are also disclosed herein. The compositions can be prepared by any one of the methods described herein. In some examples, the compositions can include doxorubicin loaded cellular vectors. The amount of doxorubicin in the cellular vector can be about 5 pg/cellular vector or greater. For example, the amount of doxorubicin in the cellular vector can be about 10 pg/cellular vector or greater, about 15 pg/cellular vector or greater, about 20 pg/cellular vector or greater, about 25 pg/cellular vector or greater, about 30 pg/cellular vector or greater, about 35 pg/cellular vector or greater, about 40 pg/cellular vector or greater, about 45 pg/cellular vector or greater, about 50 pg/cellular vector or greater, about 55 pg/cellular vector or greater, about 60 pg/cellular vector or greater, or about 70 pg/cellular vector or greater. In certain

embodiments, the amount of doxorubicin in the cellular vector can be from about 5 pg/cellular vector to about 400 pg/cellular vector, such as from about 5 pg/cellular vector to about 200 pg/cellular vector, from about 50 pg/cellular vector to about 400 pg/cellular vector, from about 50 pg/cellular vector to about 200 pg/cellular vector, from about 20 pg/cellular vector to about 100 pg/cellular vector, from about 5 pg/cellular vector to about 70 pg/cellular vector, or from about 20 pg/cellular vector to about 70 pg/cellular vector.

The cellular vectors can be washed in a wash buffer having a pH of from about 6 to about 8. The cellular vectors can be washed one, two, three, four or more times subsequent to step (c). In certain embodiments, the cellular vectors are washed in a plurality of wash buffers, wherein the wash buffer has sequentially increasing pH from about 6 to about 8. The wash buffer can include any suitable buffer solution. In some examples, the wash buffer can be a phosphate-buffered saline (PBS) solution.

The nucleated cells, after introducing the therapeutic agent (i.e., cellular vectors) becomes "non-viable." The term "viable", as used herein, refers to live cells. The term "non-viable" refers to non-living cells. Such non-viable cells may have been killed by the therapeutic agent, or otherwise inactivated, and lose their biological function, but they retain their overall size, and their ability to target and release a given payload in an organ, such as the lung, for a prolonged period of time. In some examples, substantially all of the nucleated cells become non-viable after introducing the therapeutic agent. "Substantially all" as used herein refers to 95% or greater, such as 96% or greater, 97% or greater, 98% or greater, 99% or greater, or 100%. In some examples, 98% or greater of the nucleated cells become non-viable after introducing the therapeutic agent. In some examples, all of the nucleated cells become non-viable after introducing the therapeutic agent. Methods for treating a cancerous tissue in a subject using the cellular vectors described herein are also disclosed. The method can include administering to the subject a pharmaceutically effective amount of a composition comprising the cellular vectors. In some embodiments, the cellular vectors can target the cancerous tissue after administration. In some embodiments, the cellular vectors can be locally delivered to the cancerous tissue.

Compositions comprising the cellular vectors can be administered via systemic administration, such as intravenous administration or via local administration such as by injection into the affected organ. In some examples, the composition can be administered intravenously. In certain embodiments, the methods described herein can include suspending the cellular vectors in a solution suitable for intravenous administration.

The disclosed compositions are particularly advantageous in treating pulmonary

cancer lesion or general inflammatory pathologies of the lung. Exemplary cancers which can be treated and/or imaged include, but are not limited to, lung cancer and metastases derived from other cancer diseases. Table 1 list the main sites for metastasis from other cancer diseases including skin, colorectal, ovarian, pancreatic, lung, bladder, breast, renal system, stomach, thyroid, uterus, and prostate cancer.

Table 1: Common cancer diseases and sites of metastasis.

T.1 Cancer Type	Main sites of metastasis (From NCI[4])
Bladder	Bone, Liver, Lung
Breast	Bone, Brain, Liver, Lung
Colorectal	Liver, Lung, Peritoneum
Kidney	Adrenal Gland, Bone, Brain, Liver, Lung
Lung	Adrenal Gland, Bone, Brain, Liver, other Lung
Melanoma	Bone, Brain, Liver, Lung, Skin/Muscle
Ovary	Liver, Lung, Peritoneum
Pancreas	Liver, Lung, Peritoneum
Prostate	Adrenal Gland, Bone, Liver, Lung
Stomach	Liver, Lung, Peritoneum
Tyroid	Bone, Liver, Lung
Uterus	Bone, Liver, Lung, Peritoneum, Vagina

The compositions disclosed herein contain an effective amount of doxorubicin or other therapeutic agent to treat a variety of diseases and disorders. The amount to be administered can be readily determined by the attending physician based on a variety of factors including, but not limited to, age of the patient, weight of the patient, disease or disorder to be imaged or treated, and presence of a pre-existing condition, and dosage form to be administered (e.g., immediate release versus modified release dosage form). Typically, the effective amount is from about 1 mg/kg to about 10 mg/kg of body weight, such as about 5 mg/kg of body weight. Dosages greater or less than this can be administered depending on the diseases or disorder to be treated or imaged.

After administration, the cellular vectors can accumulate in the affected tissue within about 30 minutes or less, about 25 minutes or less, about 20 minutes or less, about 15 minutes or less, about 10 minutes or less, or about 5 minutes or less. In certain embodiments, after administration, the cellular vectors can accumulate in the affected tissue, such as the lungs within about 15 minutes or less, about 10 minutes or less, about 5 minutes or less, or about 2 minutes or less, for example, within about 2 minutes to about 10

minutes.

In some examples, the cellular vectors disclosed herein exhibit rapid clearance from the blood. For example, in certain embodiments, the liver and spleen exhibit high cellular vector uptake at early time points of about 10 hours or less, about 9 hours or less, about 8 hours or less, about 7 hours or less, about 6 hours or less, about 5 hours or less, about 4 hours or less, about 3 hours or less, about 2 hours or less, or about 1.5 hour or less. In some examples, the liver and spleen exhibit high cellular vector uptake at early time points of from about 4 hours to about 8 hours or less.

EXAMPLES

The following examples are set forth below to illustrate the compositions, methods, and results according to the disclosed subject matter. These examples are not intended to be inclusive of all aspects of the subject matter disclosed herein, but rather to illustrate representative methods, compositions, and results. These examples are not intended to exclude equivalents and variations of the present invention, which are apparent to one skilled in the art.

Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight. There are numerous variations and combinations of reaction conditions, e.g., component concentrations, temperatures, pressures, and other reaction ranges and conditions that can be used to optimize the product purity and yield obtained from the described process. Only reasonable and routine experimentation will be required to optimize such process conditions.

Example 1: Cellular vectors to maximize the delivery of standard chemotherapeutics to lung metastasis

To formulate cellular vectors (CELVEC), i) an incubator, ii) an electroporator, iii) the disclosed electroporation buffer solution, iv) drug, and v) routine supplies for the isolation and growth of immune cells are used. The encapsulation of the drug can be accomplished in a short period, e.g., less than a second (**Figure 1**). Leukocytes were obtained from commercial sources, but the same protocol can be applied also to primary cells. In the case of the mice, primary macrophages can be obtained through intraperitoneal lavage or collected directly from the spleen. Drug loading through electroporation allowed for a general shrinkage of the cells due to the loss of cell turgidity. This can reduce the risk of pulmonary embolization. After CELVEC injection, the cells are entrapped in the lung vasculature where they reside for about 4 hours: after this time, about 50% of CELVEC are

cleared in liver and spleen, while after 24 hours, all the CELVEC are found in the organs. The final clearance of the cells in this organ represents a natural way that our body uses to clear and digest cellular material: in the case of CELVEC there is no formation of secondary degradation products that can be harmful for our body.

Primary leukocyte cell isolation and CELVEC loading: A group of healthy Balb/c female mice were periodically sacrificed (e.g., 40 first year, 60 second year) to isolate primary leukocytes. The leukocytes were isolated from the spleen and specific phenotypes (for example T cells and macrophages) were isolated through the use of commercial kit (Dynabeads, LifeTechnologies). The purity of the samples were confirmed through flow cytometry by analyzing the percentage of cells expressing CD3 surface markers. Primary T-cells were loaded with DOX using a 4D-NUCLEOFECTOR™ Electroporating System (LONZA) that allows for the simultaneous preparation of up to 3.5 x10^6 CELVEC per electroporation cycle (**Figure 1**). The loading and release kinetics of the CELVEC were evaluated through HPLC and fluorimetric analysis (**Figures 3A through 3C**). A continuous cell line of leukocyte (including J774, Jurkat, or THP-1) may also be used in this study.

Optimization of DOX loading: In order to better encapsulate the DOX within the cells, several washes with a physiological buffer, with increasing pH (up to 8) were performed.

Establishment of pulmonary breast cancer metastases: 4T1 mouse breast tumor cells (Perkin Elmer) were expanded *in vitro*, collected and filtered through a 40 μm strainer prior to injection. Female Balb/C mice were then injected in the tail vain with 2×10^5 cells. Preliminary data showed the formation of pulmonary breast cancer metastases in 2 weeks, whose growth was monitored through the photon emission using an IVIS 200 imager (PerkinElmer).

Evaluation of CELVEC biodistribution in tumor bearing mice: Intravital microscopy (IVM) was used to monitor the *in vivo* biodistribution of CELVEC over time. Cells were labeled with FITC through Wheat Germ Agglutinin staining prior to electroporation and injection (**Figures 4A and 4B**). Electroporated cells without incorporated DOX served as a positive control (POS-CTRL) to evaluate the effect of the drug on CELVEC behavior *in vivo*. An additional negative control (NEG-CTRL) were represented by cells previously induced for apoptosis by treatment with DOX (2 mg/mL). The NEG-CTRL was treated for different time points (2, 4, 12, 24, and 48 hours) and evaluated for Annexin V expression, which recognizes the expression of phosphatidyl serine. FACS analysis was performed to detect this parameter and to evaluate the apoptotic progression among the various

conditions and adjust the DOX treatment (time or concentration). These experiments were used to understand the impact that the apoptotic induction and DOX loading has on the membrane of CELVEC. By examining how the treatment of mice with comparable surface expression of phosphatidyl serine (i.e., NEG-CTRL) and cells without DOX (i.e., POS-CTRL), the influence of these two different unique features on the residence time and biodistribution of CELVEC can be determined.

A group of 90 mice bearing the tumor were randomly divided in 3 groups (30 mice each group) and intravenously injected with CELVEC, POS-CTRL or NEG-CTRL ($5x10^5$ CELVEC or cells stained with FITC-Wheat Germ Agglutinin per mouse). Five mice were injected with saline as a control and used to measure the background fluorescent signal. 6 mice for each group were sacrificed at five minutes, 1, 2, 4, 8, and 24 hours after CELVEC administration and the lung, liver and spleen harvested and analyzed through confocal microscopy to quantify CELVEC, NEG-CTRL or POS-CTRL. 10 images of each organ were acquired at random fields of view (n=50 for each time point for each group) to evaluate the biodistribution in the organs of interest of the different groups (**Figures 4A and 4B**).

Evaluation of DOX accumulation in lung tumor bearing mice: 60 mice were used to evaluate DOX pharmacokinetics when encapsulated in CELVEC or freely administered. Five untreated animals were used to measure the baseline. The mice were injected with 10 mg/kg/mouse of free DOX and with the same amount of drug encapsulated in CELVEC. Considering that every CELVEC can load about 400 pg of DOX and that the weight of a single mouse is around 20 g, it was estimated that an equivalent dose of DOX can be achieved by injecting approximately $0.5x10^6$ CELVEC/mouse). At the same time points described above the blood of the animals were sampled and the lung, liver and spleen harvested and homogenized. HPLC analysis were performed on all samples (**Figures 5** and **6**).

Optimization of DOX release kinetics: To maximize the delivery of DOX, CELVEC release properties can be adjusted to maximize the release of DOX in the lungs before clearance. In particular, 90% of DOX release before 50% of the CELVEC are cleared from the lung can be achieved. DOX release from the CELVEC was accelerated by enriching the electroporation solution with increasing amounts of the non-ionic surfactant Polysorbate 80. This molecule can increase the diffusion of chemotherapeutics (included DOX) through biological membranes. Varying concentrations of Polysorbate 80 (ranging from 0.0001% to 0.1 %) were added to the electroporation solution and the CELVEC analyzed for DOX

content through HPLC.

Results: Tumor growth in the lung is associated with increased inflammatory state, formation of new blood vessels, and extravasation of the blood within the lung parenchyma. The residence time of the CELVEC within the lung exceeded 4 hours, thus favoring the complete release of DOX. The comparison of POS-CTRL, loaded CELVEC, and NEG-CTRL allowed for an understanding if possible differences in the residence time of CELVEC in the lung is determined by the ongoing apoptotic process or by the high amount of DOX, that could destabilize CELVEC structure favoring its degradation and clearance. Enriching the electroporation solution with Polysorbate 80 can destabilize the cell membrane enough to accelerate the release of the payload within the intended timeframe. Evaluation of DOX content in liver and spleen (major organ accumulation of CELVEC at 24 hours) helped with the understanding of the safety of the CELVEC during clearance (Figures 5 and 6).

Alternative procedure: The electroporation procedure was modulated by increasing the power and the might of the pulse. In this case the destabilization of the cell membrane was achieved through a physical rather than a chemical way. Biological integrity and stability of the CELVEC as well as physical properties (size and shape) were evaluated for any modification of the loading procedure.

Evaluation of CELVEC inhibition of cancer growth: 40 Balb/c female mice (4 weeks old) with established lung metastases were randomly divided in 4 groups (n=10). A group of untreated mice were used to determine the baseline growth of lung metastases. The mice then received 2 injections (1 injection per week of free DOX, loaded CELVEC or empty NEG-CTRL). The efficacy of DOX-loaded CELVEC and free drug in inhibiting tumor growth were assessed in the animals as described before. Tumor growth was monitored through bioluminescent imaging (three times a week) from the beginning of the treatment to one week after the last injection. At the end of the observation period, mice were euthanized and the lungs processed for histological analysis inspecting for apoptosis (TUNEL), senescence-associated β-galactosidase activity, and proliferation (Ki-67) (Millipore). The quantification of these parameters were performed through SimplePCI imaging software.

Assessment of CELVEC safety in vivo: At the end of the experiment liver, lung, spleen and heart (recognized organ targets for DOX toxicity) were harvested, fixed in formalin and embedded in paraffin for H&E or specific staining to detect the presence of leukocytes, necrotic and apoptotic areas. A panel of 32 cytokines/chemokines/colony stimulating factors was analyzed using MILLIPLEX MAG (Millipore) ELISA assay based

on luminex magnetic beads. In order to determine complement system activation the blood was evaluated for C4d, Bb, C3adesarg, C5a, and SC5b-9 levels using ELISA commercial kits. The blood was also be tested for total white blood cells, lymphocytes, monocytes and granulocytes, red blood cell count and hemoglobin content, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean cell hemoglobin concentration, red cell distribution width, platelets count, and mean platelet volume. In addition, hepatic (ALB, ALKP, ALT, and AST) and renal (BUN, creatinine, Na^+, K^+, and Cl^-) function, and other blood parameters including CK-MB, LDH, Amyl and CHE were evaluated. This complete panel of biomarkers determined without uncertainty the safety of the CELVEC approach. During the treatment period general conditions (activity, breathing, reflection, spasm, skin and hair, eyes, discharge and secretion) were evaluated.

The animals treated with DOX-loaded CELVEC had reduced tumor burden (number and mass of metastases) than control animals and displayed histological signs of tissue recovery. Additional, emerging toxicity associated with this treatment when compared to free DOX injection was not anticipated. The comparison with the negative control allows the understanding of empty CELVEC effect on animal biology and tissue pathology. These experiments can determine the effective dose of DOX encapsulated in CELVEC ability to maximize the benefits and minimize the risks associated with this treatment.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

CLAIMS

What is claimed is:

1. A method of introducing doxorubicin into nucleated cells to form cellular vectors, comprising:
   (a) contacting a mixture comprising doxorubicin in an electroporation buffer having a pH of from about 4.5 to about 5 with the nucleated cells;
   (b) applying at least one voltage pulse between a plurality of opposing pairs of needle electrodes disposed in the mixture so as to establish an electric field in the nucleated cells sufficient to transfect doxorubicin into the nucleated cells to form the cellular vectors; and
   (c) washing the cellular vectors with a wash buffer having a pH of from about 6 to about 8,

   wherein substantially all of the nucleated cells are non-viable after step (c).

2. The method of the preceding claim, wherein doxorubicin in the mixture is at a concentration of from about 15 mg/mL to about 50 mg/mL.

3. The method of any one of the preceding claims, wherein the nucleated cells in the mixture are at a concentration of from about 1 x 10^5 cells/mL to about 1 x 10^8 cells/mL.

4. The method of any one of the preceding claims, wherein the nucleated cells comprise immune cells.

5. The method of any one of the preceding claims, wherein the nucleated cells comprise T-cells or leukocytes.

6. The method of any one of the preceding claims, wherein the voltage pulse is applied for a period of from about 1 millisecond to about 90 milliseconds.

7. The method of any one of the preceding claims, wherein the cellular vectors comprise from about 5 pg to about 400 pg doxorubicin per cellular vector.

8. The method of any one of the preceding claims, wherein the cellular vectors are sequentially washed 2 to 4 times with the wash buffer.

9. The method of any one of the preceding claims, wherein the wash buffer has sequentially increasing pH of from about 6 to about 8.

10. The method of any one of the preceding claims, wherein the electroporation buffer comprises,

(a) a zwitterionic buffering agent at a concentration of from about 1 mM to about 5 mM;

(b) a phosphate buffering agent at a concentration of from about 5 mM to about 30 mM;

(c) at least one salt, in an effective amount such that the ionic strength of the electroporation buffer is about physiological;

(d) a carbon polyol at a concentration of from about 200 mM to about 300 mM; and

(e) optionally an acid.

11. The method of any one of the preceding claims, wherein the zwitterionic buffering agent is selected from 2-(*N*-morpholino)ethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3-(*N*-morpholino)propanesulfonic acid, 3-morpholino-2-hydroxypropanesulfonic acid, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid), *N*-(2-hydroxyethyl)piperazine-*N'*-(2- hydroxypropanesulfonic acid); *N*-(2-hydroxyethyl)piperazine-*N'*-(4- butanesulfonic acid), *N*-cyclohexyl-2-aminoethanesulfonic acid, *N*-cyclohexyl-3-aminopropanesulfonic acid, 4-(cyclohexylamino)-1-butanesulfonic acid, 3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid, and combinations thereof.

12. The method of any one of the preceding claims, wherein the zwitterionic buffering agent includes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

13. The method of any one of the preceding claims, wherein the phosphate buffering agent is at a concentration of about 15 mM.

14. The method of any one of the preceding claims, wherein the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid is at a concentration of about 2 mM.

15. The method of any one of the preceding claims, wherein the carbon polyol is selected from mannitol, sorbitol, sucrose, glycerol, polyethylene glycol, xylitol, arabitol, maltitol, erythritol, isomalt, lactitol, and combinations thereof.

16. The method of any one of the preceding claims, wherein the carbon polyol is mannitol.

17. The method of any one of the preceding claims, wherein the carbon polyol is at a concentration of about 250 mM.

18. A composition prepared according to any one of the methods of claims 1-17.

19. A method of treating a cancerous tissue in a subject comprising, administering to the subject a pharmaceutically effective amount of a composition according to claim 18.

20. The method of the preceding claims, wherein the composition is administered intravenously.

21. The method of any one of the preceding claims, wherein the cancerous tissue is in the lung.

22. An electroporation buffer solution, comprising:
    (a) a zwitterionic buffering agent at a concentration of from about 1 mM to about 5 mM;
    (b) a phosphate buffering agent at a concentration of from about 5 mM to about 30 mM;
    (c) at least one salt, in an effective amount such that the ionic strength of the

solution is about physiological;

(d) a carbon polyol at a concentration of from about 200 mM to about 300 mM; and

(e) optionally an acid,

wherein the pH of the electroporation buffer solution is from about 4.5 to about 5.

23. The electroporation buffer solution of the preceding claim, wherein the zwitterionic buffering agent is selected from 2-(*N*-morpholino)ethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3-(*N*-morpholino)propanesulfonic acid, 3-morpholino-2-hydroxypropanesulfonic acid, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid), *N*-(2-hydroxyethyl)piperazine-*N'*-(2- hydroxypropanesulfonic acid); *N*-(2-hydroxyethyl)piperazine-*N'*-(4- butanesulfonic acid), *N*-cyclohexyl-2-aminoethanesulfonic acid, *N*-cyclohexyl-3-aminopropanesulfonic acid, 4-(cyclohexylamino)-1-butanesulfonic acid, 3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid, and combinations thereof.

24. The electroporation buffer solution of any one of the preceding claims, wherein the zwitterionic buffering agent includes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

25. The electroporation buffer solution of any one of the preceding claims, wherein the phosphate buffering agent is at a concentration of about 15 mM.

26. The electroporation buffer solution of any one of the preceding claims, wherein 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid is at a concentration of about 2 mM.

27. The electroporation buffer solution of any one of the preceding claims, wherein the carbon polyol is selected from mannitol, sorbitol, sucrose, glycerol, polyethylene glycol, xylitol, arabitol, maltitol, erythritol, isomalt, lactitol, and combinations thereof.

28. The electroporation buffer solution of any one of the preceding claims, wherein the carbon polyol is mannitol.

29. The electroporation buffer solution of any one of the preceding claims, wherein the carbon polyol is at a concentration of about 250 mM.

30. The electroporation buffer solution of any one of the preceding claims, wherein the electroporation buffer solution further comprises a surfactant.

31. The electroporation buffer solution of any one of the preceding claims, wherein the surfactant is a polysorbate surfactant.

32. A kit comprising an electroporation buffer solution according to any one of the preceding claims.

33. The kit of the preceding claim, further comprising a therapeutic agent.

34. The kit of any one of the preceding claims, wherein the therapeutic agent is a chemotherapeutic agent.

35. The kit of any one of the preceding claims, wherein the chemotherapeutic agent is doxorubicin or daunomycin.

36. The kit of any one of the preceding claims, wherein the electroporation buffer solution and the therapeutic agent are provided in separate containers.

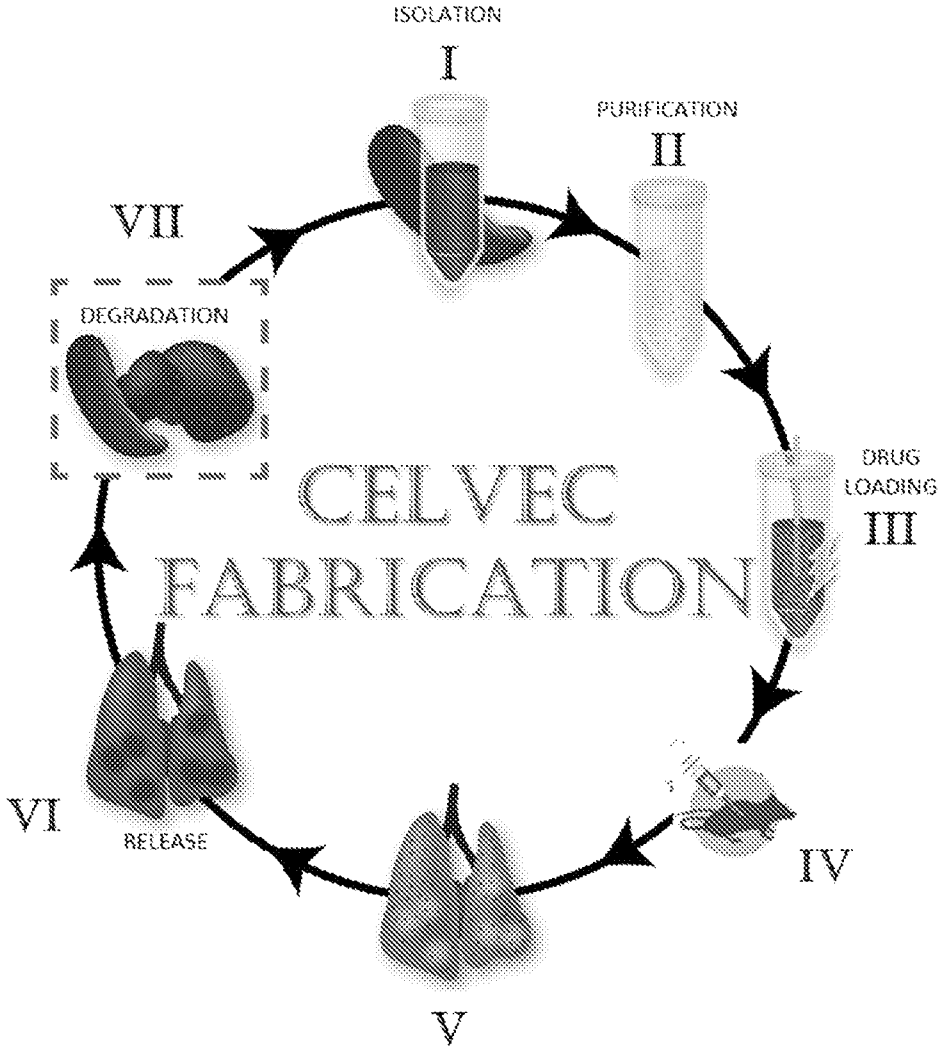
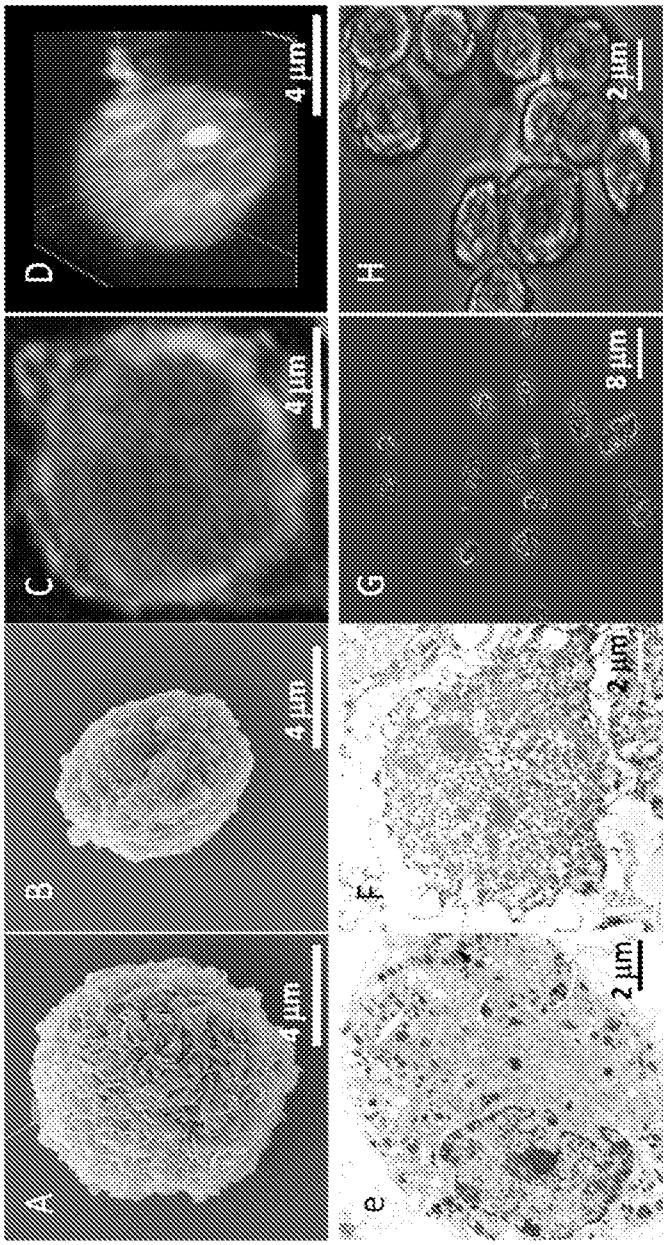

ISOLATION
I
PURIFICATION
II
DRUG
LOADING
III
IV
V
VI
RELEASE
VII
DEGRADATION
CELVEC
FABRICATION

FIG. 1



FIGS. 2A-2H

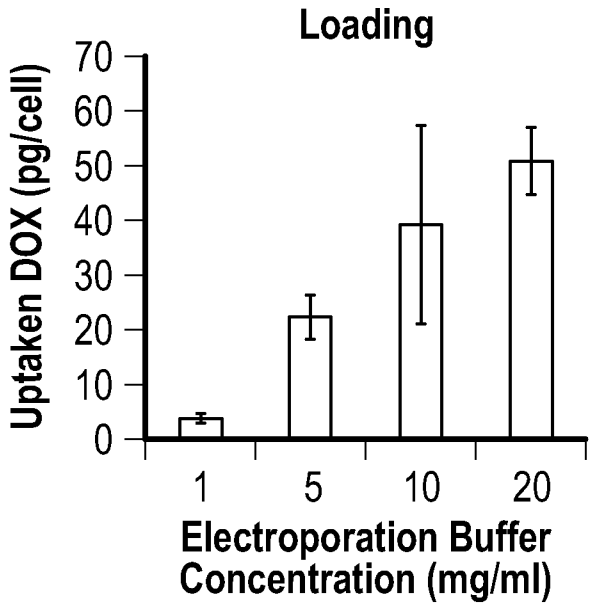


FIG. 3A

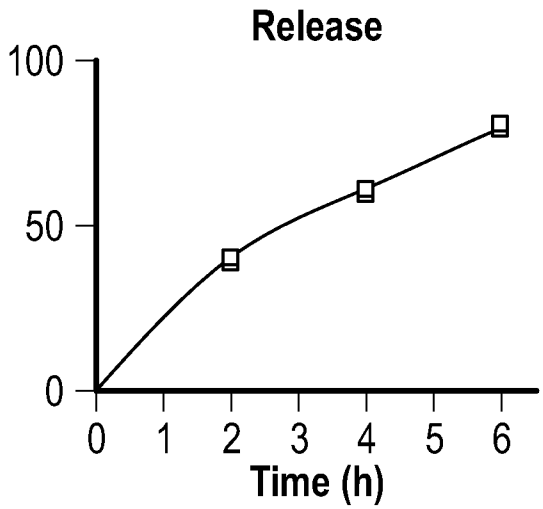


FIG. 3B

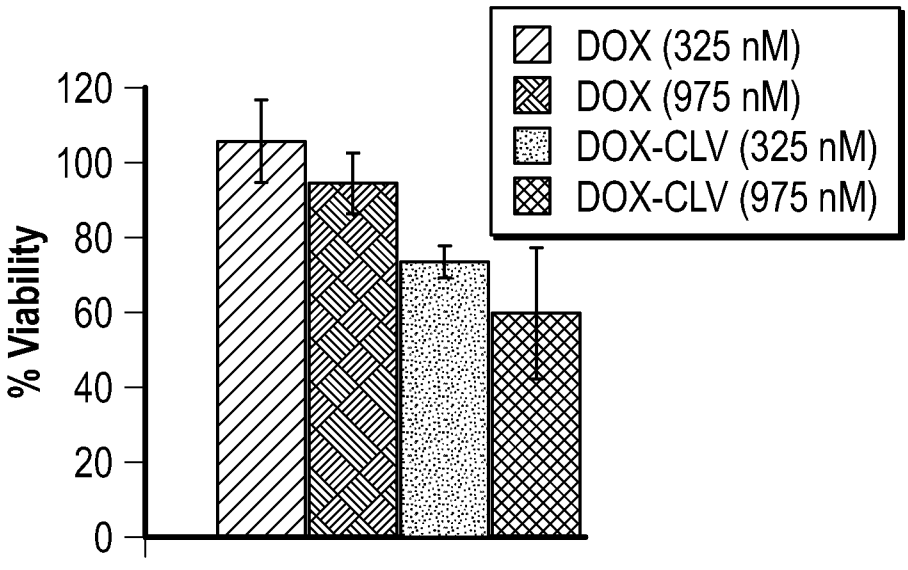


FIG. 3C

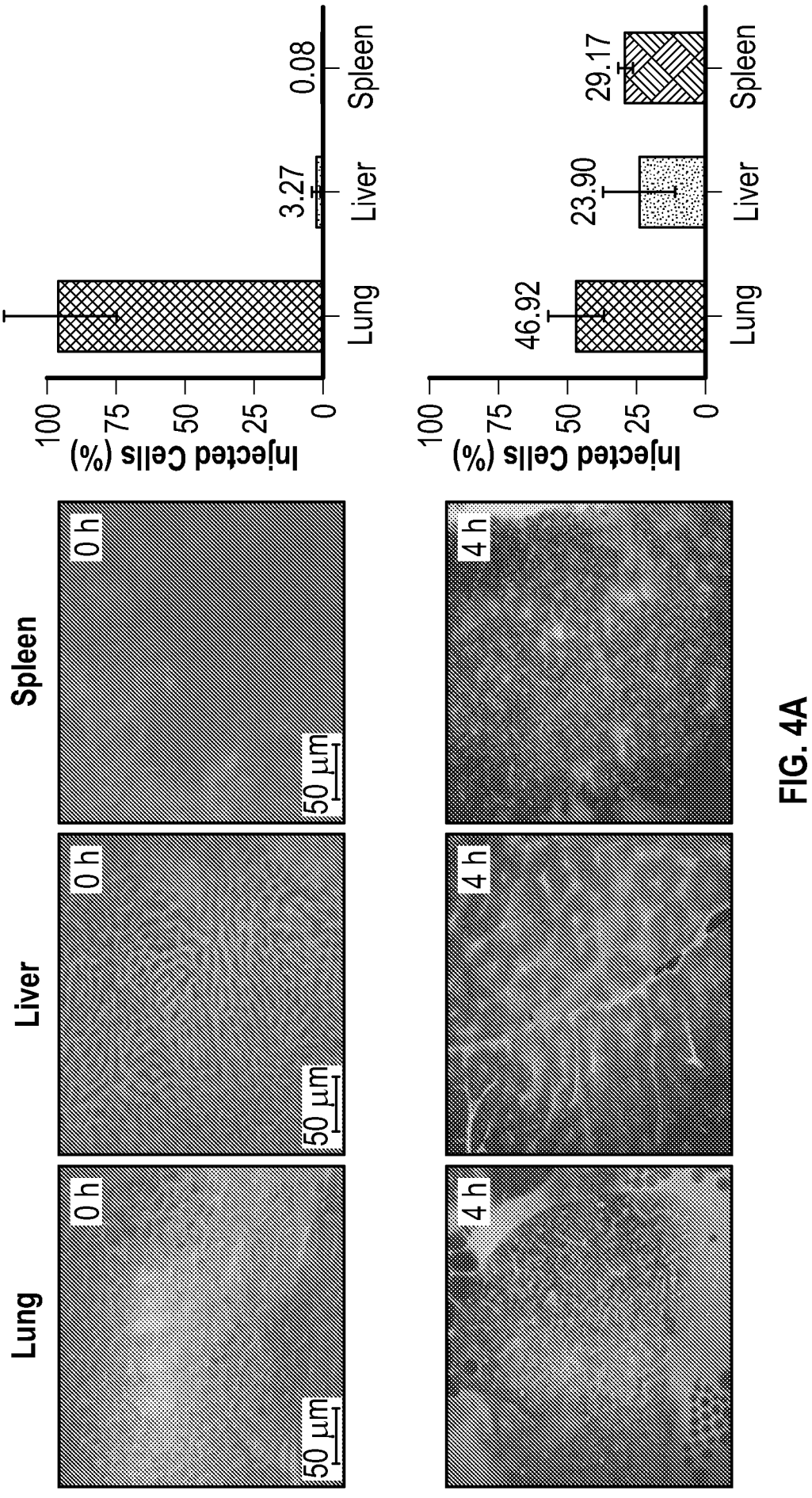

Lung
Liver
Spleen
0 h
4 h
50 μm
Injected Cells (%)
100
75
50
25
0
96.65
3.27
0.08
46.92
23.90
29.17
Lung
Liver
Spleen

FIG. 4A

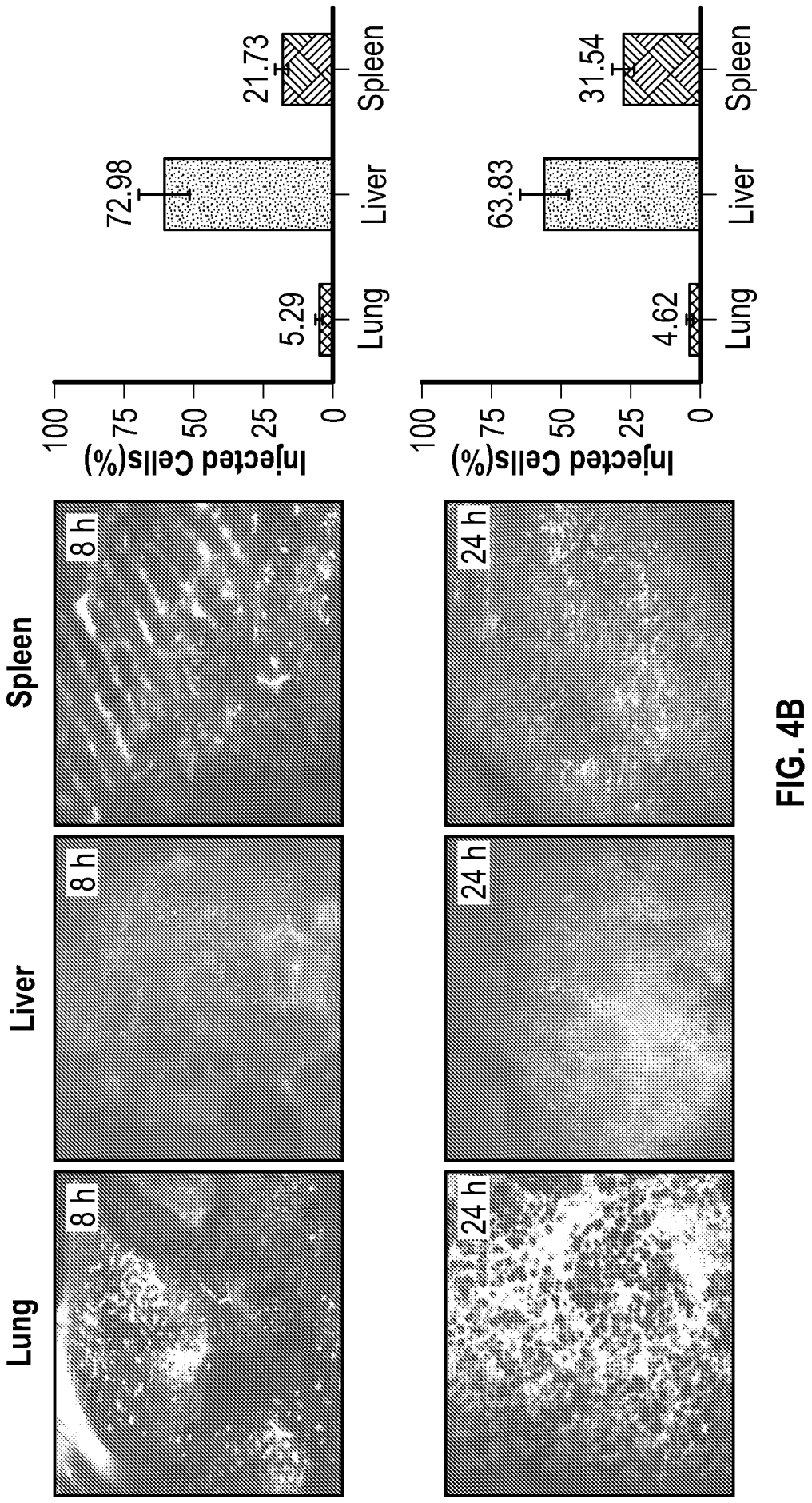

Lung
Liver
Spleen
8 h
24 h
Injected Cells(%)
100
75
50
25
0
5.29
72.98
21.73
4.62
63.83
31.54
Lung
Liver
Spleen

FIG. 4B

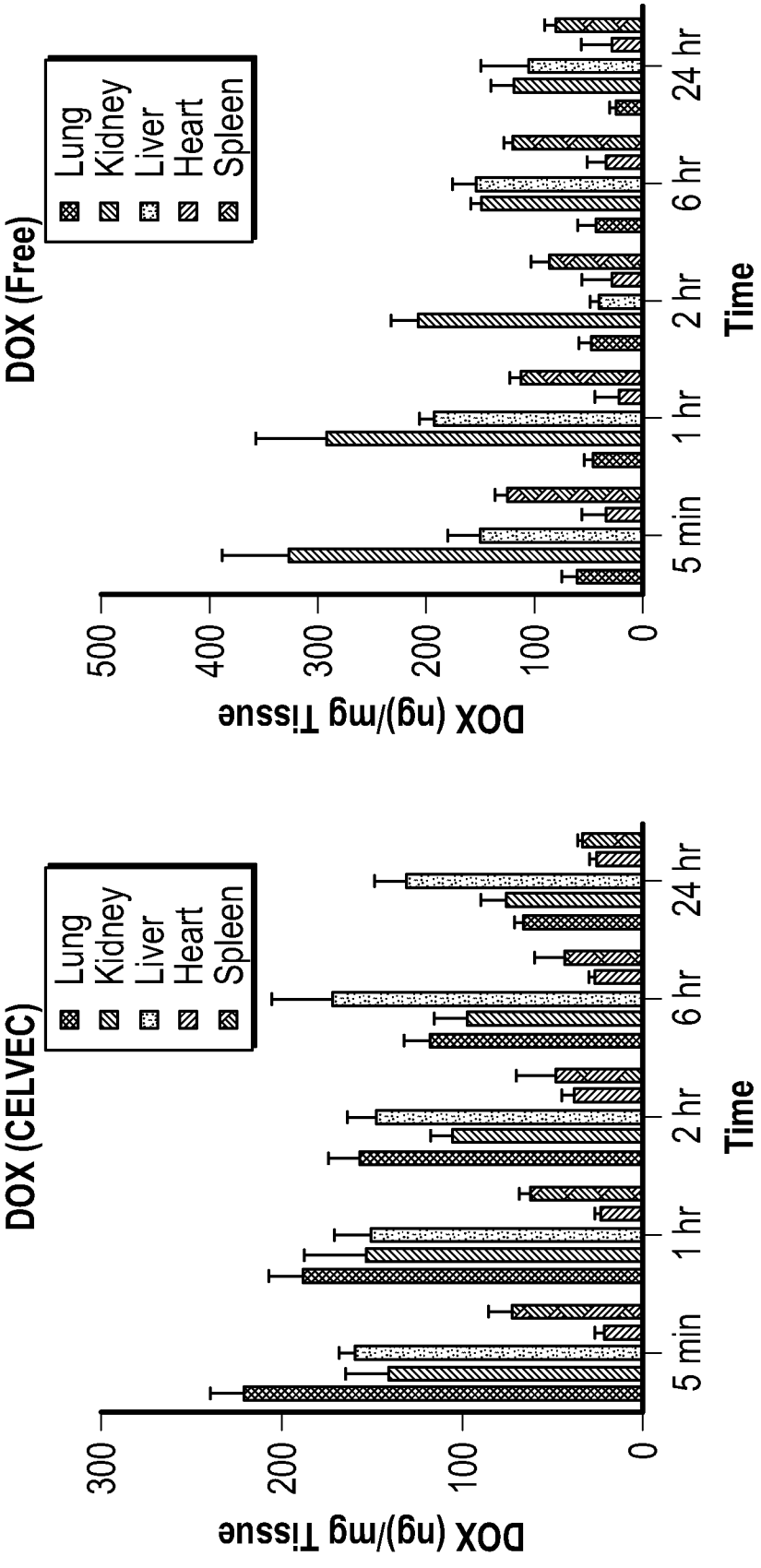

DOX (Free)
Lung
Kidney
Liver
Heart
Spleen
DOX (ng)/mg Tissue
500
400
300
200
100
0
5 min
1 hr
2 hr
6 hr
24 hr
Time
DOX (CELVEC)
Lung
Kidney
Liver
Heart
Spleen
DOX (ng)/mg Tissue
300
200
100
0
5 min
1 hr
2 hr
6 hr
24 hr
Time

FIG. 5

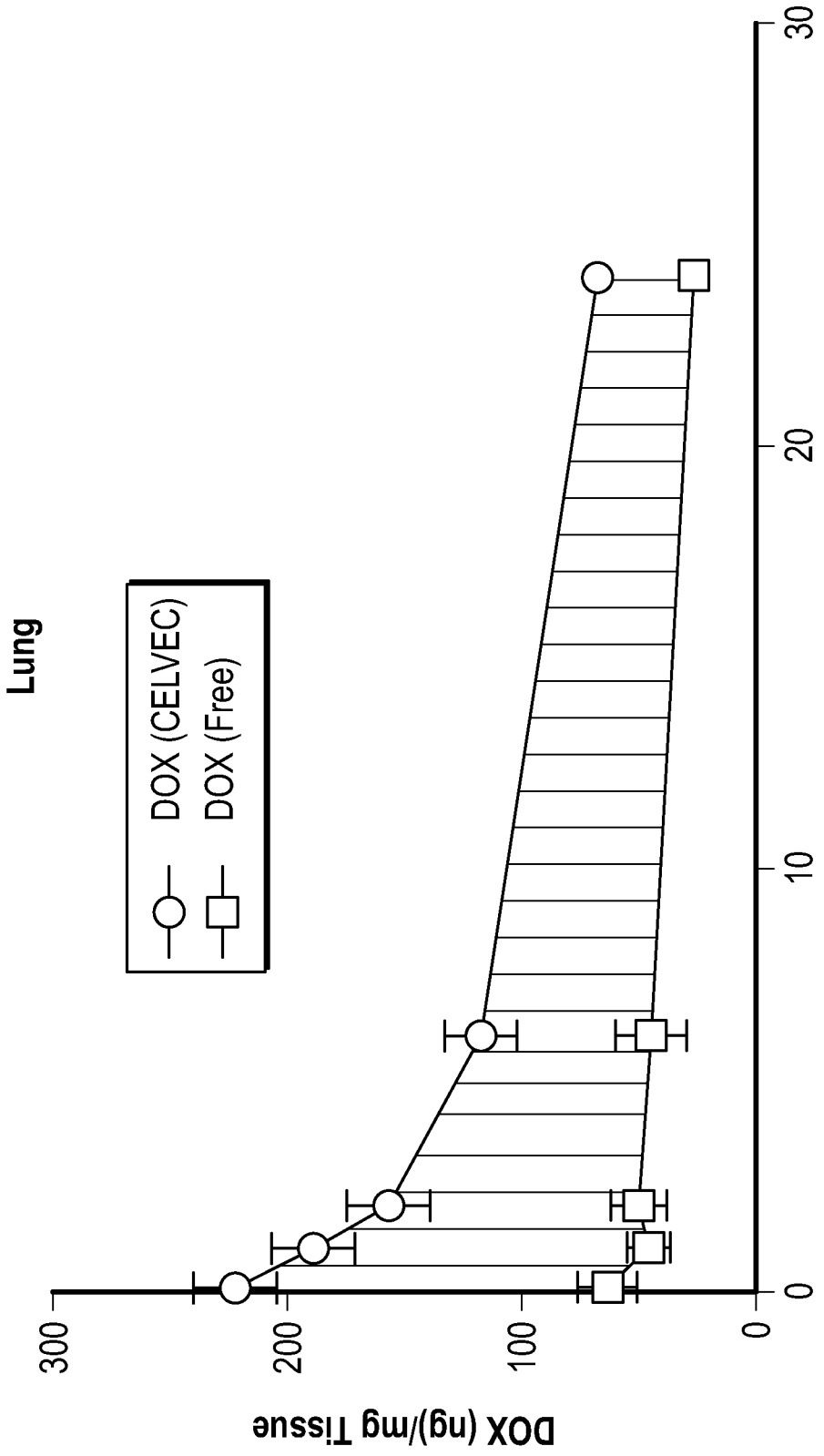

Lung
DOX (CELVEC)
DOX (Free)
DOX (ng)/mg Tissue
300
200
100
0
0
10
20
30
Time (hour)

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/57679

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/078; A61P 35/02, 35/04 (2016.01)
CPC - C12M 35/02; A61N 1/327; A61K 9/5068, 9/0009
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 5/078; A61P 35/02, 35/04 (2016.01);
CPC - C12M 35/02; A61N 1/327; A61K 9/5068, 9/0009

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA); Google Scholar; EBSCO; PubMed; Doxorubicin, Adriamycin, Doxil, Caelyx, Myocet, nucleated cell, electroporation, electropermealbilization, electric field, wash, rinse, buffer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/35389 A1 (NEDERLANDSE ORGANISATIE VOOR TOEGEPAST-NATUURWETENSCHAPPELIJK ONDERZOEK TNO) 28 December 1995; page 12, lines 35-38; page 15, lines 17-18; page 16, lines 26-33; page 18, lines 36-38; page 19, lines 1-9; page 21, lines 11-13	1-2, 22-23
Y	US 2011/0236979 A1 (BEEBE, SJ et al.) 29 September 2011; paragraphs [0037], [0040], [0045], [0072]-[0074], [0077], [0079], [0091], [0099]	1-2
Y	US 6,014,584 A (HOFMANN, GA et al.) 11 January 2000; column 3, lines 39-45; column 10, lines 39-41; column 11, lines 16-18; column 21, lines 24-26	1-2
Y	US 6,589,786 B1 (MANGANO, JA et al.) 08 July 2003; column 12, lines 13-18; column 16, lines 54-55; column 52, lines 27-42; claim 1	1-2
Y	(IDANI, H et al.) Intra-tumoral Injection of Doxorubicin (Adriamycin) Encapsulated In Liposome Inhibits Tumor Growth, Prolongs Survival Time And Is Not Associated With Local Or Systemic Side Effects. International Journal of Cancer. 2000. vol. 88; abstract; page 645, seventh paragraph; page 647, fifth paragraph	2
Y	US 2009/0197335 A1 (AGRAWAL, DK et al.) 06 August 2009; abstract; paragraphs [0033], [0035]; claim 1	22-23
A	WO 02/07752 A2 (GENDEL LIMITED) 31 January 2002; entire document	1-2, 22-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 December 2016 (07.12.2016)	26 JAN 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/57679

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 3-21 and 24-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.