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| <b>(54) Title:</b> NUCLEIC ACID DELIVERY                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| <b>(57) Abstract</b><br><br>The invention relates to a method for delivering a nucleic acid to a target site in an organism, comprising the steps of: a) loading a red blood cell with a nucleic acid; b) introducing into the organism the red blood cell loaded with the nucleic acid; and c) causing the nucleic acid to be released from the red blood cell at the target site in the organism.                                                                                                                                                                                                                                                                                                                                                                                                                                   |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |

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## NUCLEIC ACID DELIVERY

The present invention relates the delivery of nucleic acids to cells and/or tissues. In particular, the invention relates to the delivery of nucleic acids using erythrocytes as  
5 delivery vehicles, and to the photosensitisation of erythrocytes to permit selective disruption thereof by photodynamic activation.

The delivery nucleic acids to specific cells or tissues is desirable in gene therapy and other nucleic acid-based therapies to ensure that a sufficiently high dose of a given nucleic acid  
10 is delivered to a selected cell or tissue. Moreover, it is often the case that the nucleic acid, although advantageously having beneficial therapeutic effects on the diseased tissue, may have undesirable side effects on tissues that are not diseased. This is typified by, but not limited to, nucleic acids which encode cancer prodrugs that destroy tumour cells upon  
15 activation. Although cancer cells which express the prodrug may be destroyed, non-cancerous cells which have also taken up a nucleic acid which encodes the prodrug may also be affected resulting in cell death and severe side effects. It is, therefore, desirable to provide a carrying means for the delivery of nucleic acids to selected tissues to reduce the non-specific effects of said nucleic acids.

20 Attempts to improve delivery of nucleic acids include the use of monoclonal antibodies to cell surface antigens of selected tissues. It has been known for many years that tumour cells produce a number of tumour cell specific antigens, some of which are presented at the tumour cell surface. Monoclonal antibodies generated to these antigens are considered useful targeting agents for nucleic acid delivery. However, clinical trials have  
25 generally been disappointing. The major reason for this lack of therapeutic efficacy is the lack of penetration of large immunoglobulin/DNA complexes. Reasons for poor penetrative ability of antibodies include the inability to cross endothelial membranes and densely-packed tumour cells within a tumour cell mass.

30 Alternative means to deliver, or carry, nucleic acids comprise the use of synthetically manufactured liposomes. These are lipid based vesicles which encapsulate a selected nucleic acid which is then introduced into a patient. The liposome is manufactured either

from pure phospholipid or a mixture of phospholipid and phosphoglyceride. Typically liposomes can be manufactured with diameters of less than 200nm. This enables them to be intravenously injected and to pass through the pulmonary capillary bed. Furthermore, the biochemical nature of liposomes confers permeability across blood vessel membranes to gain access to selected tissues. The use of liposomes as drug delivery vehicles is described in U.S. Patent No. 5,580,575 and U.S. Patent No. 5,542,935.

Despite their advantages, liposomes suffer from major drawbacks. First, liposomes lack intrinsic affinity for the targeted tissues and rely on a local intravenous injection of the liposome composition in the vicinity of the diseased tissue. Second, they have a relatively short half-life when introduced into an individual. Finally, liposomes are not "immune-silent" and may, therefore, induce an immune response from the patient. This is a major disadvantage if long term treatment is required, as is the case in many cancers.

To address the issue of liposome specificity, immunoliposomes have been developed which combine the drug containing liposome with a selected antibody to improve localisation, but the problems presented when using cytotoxic monoclonal antibodies, as described above, are also evident when using immunoliposomes. In order to increase the relatively short half-life and immune detection of liposomes, so called STEALTH® liposomes have been developed which comprise liposomes coated in polyethylene glycol (PEG). The PEG-treated liposomes have a significantly increased half-life when administered intravenously to a patient. In addition, STEALTH® liposomes show reduced uptake in the reticuloendothelial system and enhanced accumulation in, for example, tumours. STEALTH® liposomes have also been combined with monoclonal antibodies to improve specificity. However it has been reported that although STEALTH® liposomes have increased biological retention there are adverse side effects including stomatitis, palmar-plantar erythrodysesthesia, nausea, vomiting and alopecia.

A further example of a means to deliver or carry a nucleic acid comprises human gene therapy vectors derived from viruses. The rationale for this approach is that such vectors can easily penetrate cells by virtue of their ability to naturally infect human cells and so can incorporate foreign DNA into a target cell population.

A nucleic acid vector comprising a gene of interest can be incorporated into liposomes or, alternatively, can be complexed with various lipids to facilitate DNA transfer to cells. For example, a lipopolyamine may be complexed with vector DNA to form lipid based micelles (Pitard et al., 1997, Proc. Natl. Acad. Sci. U.S.A., 94: 14412-14417). The lipopolyamine/DNA complex condenses into spherical particles having a diameter of approximately 50 nm. In addition, polyethylenimine (PEI) has been shown to be an effective condensing agent for adenovirus particles. The condensed virus/PEI complex is approximately 100nm in diameter and is effectively delivered to cells without the requirement of viral infectivity, while the virus is psoralen inactivated to prevent viral gene expression (Baker et al., 1997, Gene Therapy, 4: 773-782).

Alternative nucleic acid condensing agents include peptides. For example, 18-mer peptides containing lysine have been utilised both as condensing agents and as inhibitors of serum nuclease attack of a nucleic acid vector. It will be apparent therefore that the delivery of gene therapy vectors to selected tissues is problematic since the delivery agent has to not only deliver the vector DNA to the selected tissue, but also protect the DNA from the patient's nucleases found intracellularly and in serum.

The most widely used viruses are of the Adenovirus, Retrovirus, Parvovirus and Herpesvirus families. These viruses can be genetically manipulated to render them incapable of replication to prevent the spread of viral infection to other tissues. However, in many cases these vectors do result in tissue damage and respiratory disease (see Mulligan, 1993, Science, 260: 926-932). Moreover, viral vectors are not immunoprivileged, thus leading to an immune challenge by the patient and clearing of vectors in subsequent rounds of treatment. Alternatively a number of plasmid based vectors exist that provide constitutive or regulated expression.

Erythrocytes have been proposed as vehicles for the delivery of active agents in biomedical applications (Chalmers, R.A., 1985, Bibl. Haematol. 51, 15-24). However, effective methods for targeting erythrocytes have not been proposed to date.

When packaging carrier/delivery systems such as liposomes or erythrocytes are used in *in vivo* scenarios, the delivery function is dependant upon accumulation and breakdown of the membrane in the relevant tissue/site unless a specific mechanism is incorporated into the vehicle to accommodate such accumulation and release of the relevant vehicle load.

5 It has been proposed that biospecific interactions might be exploited in achieving accumulation at a specific site, for example, the use of antibody-antigen interactions where the antibody is incorporated onto the surface of the vehicle membrane. Many such mechanisms however are precluded when considering erythrocytes as a result of the mass of the vehicle.

10

Photodynamic activation is dependant upon the observation that when certain molecules known as photosensitisers, for example hematoporphyrin derivative (HPD), are irradiated with light an event occurs which results in electrons being raised to higher energy levels. When these electrons relax to the ground state the resultant energy released is transferred to molecular oxygen resulting in a splitting of that molecule to produce what is referred to as 'singlet oxygen'. This is a highly reactive species and if the photosensitiser was incorporated into biological membranes during photoactivation, then the singlet oxygen would oxidise the lipids with resultant destruction of membrane integrity. This forms the basis for photodynamic therapy, a relatively novel treatment modality for cancer and other disorders described by Doughty, T.J. *et al.*, (1984, In "Porphyrin Localisation and Treatment of Tumours" Eds: Doirin, D.R. & Gower, C.J., Published by Alan R. Liss, N.Y. p 302). Photodynamic activation has not, however, been used to date for the delivery of nucleic acids, because it would have been expected that photodynamic energy would disrupt nucleic acid molecules.

25

### Summary of the Invention

Although erythrocytes have been used to deliver chemical agents, there have been no reports of the delivery of nucleic acids to cells or tissues such that they are expressed therein using such vehicles.

30

The invention thus provides a method for delivering a nucleic acid to a target site in an organism, comprising the steps of:

- a) loading a red blood cell with a nucleic acid;
- b) introducing into the organism the red blood cell loaded with the nucleic acid; and
- 5 c) causing the nucleic acid to be released from the red blood cell at the target site in the organism.

As used herein, the term "red blood cell" refers to a living, enucleate erythrocyte (i.e., a mature erythrocyte) of a vertebrate. Preferably, the vertebrate is a mammal.

10

As used herein, the term "cell" refers to a viable, naturally-occurring or genetically engineered, single unit of an organism.

As used herein, the term "target" is used in reference to the spatial coordinates (anatomical location) of the site, tissue or cell to which a nucleic acid is delivered according to the invention.

As used herein, the term "site" refers to a region of the body of an organism, which region may comprise an anatomical area, a tissue, a group of tissues, a cell, a group of cells or even substantially all of the cells of the organism. As used herein, the term "organism" refers to all cellular life-forms, such as prokaryotes and eukaryotes, as well as non-cellular, nucleic acid-containing entities, such as bacteriophage and viruses. Preferably, an organism is a mammal. As used herein, the term "mammal" refers to a member of the class Mammalia including, but not limited to, a rodent, lagomorph, pig or primate. In preferred embodiments, the term "mammal" refers to a human.

As used herein, the term "tissue" refers to a population or physical aggregation of cells within an organism, wherein the cells are of the same cell type or are of cell different types resident within a single organ or other functional unit. As used herein, the term "tissue" refers to intact tissue or tissue fragments, such that the cells are sufficiently aggregated (associated) so as to form a cohesive mass. Alternatively, the term "tissue" refers to a collection of individual cells, such as those which circulate (e.g., in blood or

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lymphatic fluid) within the mammal. A tissue may comprise an entire organ (e.g. the pancreas, the thyroid, a muscle, bone or others) or other system (e.g. the lymphatic system) or a subset of the cells thereof; therefore, a tissue may comprise 0.1-10%, 20-50% or 50-100% of the organ or system (e.g., as is true of islets of the pancreas).

5

As used herein, the term "loading" refers to causing a nucleic acid to become internalised by, affixed to the surface of or anchored into the plasma membrane of a red blood cell. Such loading may be performed by methods such as are described below, including, but not limited to, chemical crosslinking, osmosis, osmotic pulsing, mechanical  
10 perforation/restoration of the plasma membrane by shearing, single-cell injection and electroporation or a combination thereof. Electroporation is a highly preferred method for loading nucleic acids into a red blood cell according to the invention. It will be appreciated by one of skill in the art that combinations of methods may be used to facilitate the loading of a red blood cell with nucleic acids according to the invention.  
15 Likewise, it will be appreciated that two or more nucleic acids may be loaded concurrently or sequentially, in any order, into a red blood cell in a method of the invention.

It is preferred that the loading is performed by a procedure selected from the group  
20 consisting of electroporation, microinjection, membrane intercalation, microparticle bombardment, lipid-mediated transfection, viral infection (e.g., with a parvovirus), osmosis, osmotic pulsing, endocytosis and crosslinking to a red blood cell surface component or a combination thereof.

25 A red blood cell which comprises two or more nucleic acids is said herein to be "co-loaded". Co-loading of a red blood cell with nucleic acids may be performed such that the nucleic acids are loaded individually (in sequence) or together (simultaneously or concurrently), in the latter case regardless of whether the two nucleic acid are first admixed at the time of contact with the red blood cells or prior to that time.

30

Nucleic acids loaded into a red blood cell for use in the invention may be referred to as the "payload" of that cell. The term "payload" does not refer to the naturally-occurring contents of a red blood cell.

5 As used herein in reference to administration of a red blood cell to an organism, the term "introducing" refers to causing the red blood cell to enter the circulatory system of the organism by transfusion. Where the organism is a vertebrate, such as a mammal, it is contemplated that a hollow needle, such as a hypodermic needle or cannula, is inserted through the wall of a blood vessel (e.g., a vein or artery) and the red blood cell is either  
10 injected using applied pressure or allowed to diffuse or otherwise migrate into the blood vessel. It is understood that the diameter of the needle is sufficiently large and the pressure sufficiently light to avoid damage of the cell by shear forces. Preferably, introduction of a red blood cell into a mammal in a method of the invention is intra-arterial or intravenous. Methods of blood cell transfusion are well known in the art.

15 It is preferred that the red blood cell which comprises nucleic acid is contacted with a sensitising agent prior to the step of introducing the cell into the mammal. As used herein, the term "sensitising agent" refers to a substance which renders a cell membrane susceptible to disruption, such that the cell is susceptible to lysis or leakage of contents,  
20 by a force which would not otherwise produce such disruption, lysis or leakage. Sensitising agents of use in the invention include, but are not limited to, a hematoporphyrin derivative (HPD) and Photofrin®, a photo-activatable adapted porphyrin which binds to cell membranes.

25 As used herein, the term "nucleic acid" is defined to encompass DNA and RNA or both synthetic and natural origin which DNA or RNA may contain modified or unmodified deoxy- or dideoxy- nucleotides or ribonucleotides or analogues thereof. The nucleic acid may exist as single- or double-stranded DNA or RNA, an RNA/DNA heteroduplex or an RNA/DNA copolymer, wherein the term "copolymer" refers to a single nucleic acid  
30 strand that comprises both ribonucleotides and deoxyribonucleotides.

The term "synthetic", as used herein, is defined as that which is produced by in vitro chemical or enzymatic synthesis.

Preferably, the nucleic acid encodes a biological effector molecule. As used herein, the  
5 term "biological effector molecule" refers to an agent that has activity in- or upon a cell, including, but not limited to, a protein, polypeptide or peptide, including, but not limited to, a structural protein, an enzyme, an antibiotic, a polyclonal or monoclonal antibody, or an effective part thereof, such as an Fv fragment, which antibody or part thereof may be natural, synthetic or humanised, a peptide hormone, a receptor, a signalling molecule or  
10 other protein, or a biologically active nucleic acid such as a ribozyme or antisense molecule.

Particularly useful classes of biological effector molecules include, but are not limited to, antibiotics, anti-inflammatory drugs, angiogenic or vasoactive agents, growth factors and  
15 cytotoxic agents (e.g., tumour suppressers). Cytotoxic agents of use in the invention include, but are not limited to, diphtheria toxin, Pseudomonas exotoxin, cholera toxin and pertussis toxin, an activating polypeptide which converts an inactive prodrug to active drug form, and which activating polypeptide is selected from the group that includes, but is not limited to, viral thymidine kinase (encoded by Genbank Accession No. J02224),  
20 carboxypeptidase A (encoded by Genbank Accession No. M27717),  $\alpha$ -galactosidase (encoded by Genbank Accession No. M13571),  $\beta$ -glucuronidase (encoded by Genbank Accession No. M15182), alkaline phosphatase (encoded by Genbank Accession No. J03252 J03512), or cytochrome P-450 (encoded by Genbank Accession No. D00003 N00003), plasmin, carboxypeptidase G2, cytosine deaminase, glucose oxidase, xanthine  
25 oxidase,  $\beta$ -glucosidase, azoreductase, t-gutamyl transferase,  $\beta$ -lactamase, or penicillin amidase. Preferably, the polypeptide capable of activating a prodrug is DT diaphorase.

According to the invention, nucleic acids which are loaded into a red blood cell are released from the red blood cell and into their surroundings, in this case at or into the  
30 target site, tissue or cell, by the application of an energy pulse focused on a target site, tissue or cell. Preferably, the energy pulse is a pulse of photodynamic energy, such as laser energy.

Preferably, the nucleic acids are caused to be released from the cell via disruption of the cell.

- 5 In a preferred embodiment, the nucleic acids are caused to be released from the cell by treatment of the target site with light.

Advantageously, the invention provides a kit comprising one or more components necessary for the practice of the invention, packaging materials and, optionally,  
10 instructions for use.

Preferably, the invention provides a kit comprising a red blood cell, a nucleic acid and packaging materials therefor. In a preferred embodiment, the red blood cell is loaded with the nucleic acid.

15 The kit according to the invention may further comprise a sensitising agent and a liquid selected from the group consisting of a buffer, diluent or other excipient. Preferably, the liquid is selected from the group consisting of a saline buffer, a physiological buffer and plasma.

20 As used herein, the term "physiologically compatible buffer" or "physiological buffer" is defined as a liquid composition which, when placed in contact with living cells, permits the cells to remain alive over a period of minutes, hours or days. As such, a physiological buffer is substantially isotonic with the cell, such that cell volume does not change more  
25 than 20% due to differences in internal and external ionic strength. Non-limiting examples of physiologically compatible buffers or physiological buffers include dilute saline, which may be buffered (e.g., Hanks' buffered saline or phosphate buffered saline), or other physiological salts (e.g., Ringer's solution), dilute glucose, sucrose or other sugar, dilute glycerol with- or without salts or sugars, cell culture media as are known in the art,  
30 serum and plasma.

Preferably, the red blood cell of the physiological composition is human.

### Brief Description of the Drawings

5 Figure 1 represents the association of pAR2 (bacterial expression vector) with human red blood cells.

Figure 2 represents agarose gel electrophoresis of pAR2 extracted from E.coli cells transformed with photo-released plasmid DNA from human red blood cells.

10

Figure 3 represents the association of pSV- $\beta$ -galactosidase (mammalian expression vector) with human red blood cells.

15 Figure 4A presents a photomicrograph of control CHO cells. Fig. 4B depicts CHO cells transformed with pSV- $\beta$ -galactosidase showing transformation foci. Figs. 4C and 4D show CHO cells before and after electroporation in the presence of photo-released plasmid DNA, respectively.

20 Figure 5 shows a photograph of an agarose gel electrophoretic analysis of *Acc* I-digested DNA (lane 2) extracted from erythrocytes co-loaded with pAR2 and pSV- $\beta$ -gal.

### Detailed Description of the Invention

25 The invention provides a means for the efficient delivery from a single cell (red blood cell) of a nucleic acid to a target site, tissue or cell. The invention therefore provides a means for targeted delivery of a nucleic acid to a selected tissue in an organism.

#### A. Cells of Use in the Invention

30

Red blood cells which may be loaded with nucleic acid and administered to an organism according to the invention are ideally obtained from the intended recipient individual prior

to the procedure so as to ensure complete immunocompatibility. Alternatively, cells are obtained from a second individual of the same species as the recipient; in such a case, the second individual preferably shares the blood type of the intended recipient or must have an immuno-neutral blood type, such as type O in humans.

5

## **B. Loading a Red Blood Cell for Use in the Invention**

### i. Electroporation

10 In the Examples below, red blood cells are loaded with nucleic acid(s) using electroporation. According to this method, a momentary exposure of a cell sample to a high electric field results in transitory membrane permeabilisation. The strength of the electric field is adjusted up or down depending upon the resilience or fragility, respectively, of the cells being loaded and the ionic strength of the medium in which the  
15 cells are suspended. This method, which facilitates the passage of nucleic acids into the cell without significant loss of cellular contents or cell viability, is well known in the art. The procedure is briefly summarised as follows:

Electroporative loading is carried out by delivering single pulses of electric current to  
20 samples of cells suspended together with the relevant payload in 0.1 to 1 ml of a physiological buffer, as defined above. Each sample is placed in an electroporation cuvette (typically having a volume 0.2 to 2 ml) with an electrode gap of from 0.05 to 0.4 cm. Cuvettes are connected to an electric power source, such as a BioRad Gene Pulser. The desired voltage and capacitance are preset prior to firing of the electric pulse;  
25 alternatively, if a unit set to deliver a single energy level is used, the duration of the pulse instead can be adjusted. It is well within the knowledge of one of skill in the art to make such an adjustment. Unless otherwise stated, samples to be electroporated are placed on ice for a brief period of time (e.g., 5 minutes to one hour, typically about 10 minutes) prior to delivery of the pulse. After delivery of the pulse, samples are allowed to rest on ice to  
30 reseal cell membranes for at least 1 hour.

It will be apparent to one skilled in the art that additional methods to load erythrocytes are known. These several techniques may be briefly summarised as follows:

ii. Osmotic pulsing

5

The "osmotic pulse" mechanism is taught in U.S. Pat. No. 4,478,824. That method involves incubating a packed red blood cell fraction in a solution containing a compound (such as dimethyl sulphoxide or glycerol) which readily diffuses into and out of cells, rapidly creating a transmembrane osmotic gradient by diluting the suspension of RBC in the solution with a near-isotonic aqueous medium. This medium contains an anionic agent to be introduced (such as a phosphorylated inositol) which may be an allosteric effector of haemoglobin, thereby causing diffusion of water into the cells with consequent swelling thereof and increase in permeability of the outer membranes of the cells. This increase in permeability is maintained for a period of time sufficient only to permit transport of the anionic agent into the cells and diffusion of the readily-diffusing compound out of the cells. This method is of limited effectiveness where the desired agent to be loaded into cells is not anionic, or is anionic or polyanionic but is not present in the near-isotonic aqueous medium in sufficient concentration to cause the needed increase in cell permeability without cell destruction.

20

U.S. Patent No. 4,931,276 and WO 91/16080 disclose methods of loading red blood cells with selected agents; therefore, these techniques can be used to enable loading of erythrocytes in the present invention. In U.S. Patent No. 4,931,276, the osmotic pulse technique is modified as follows: An intact erythrocyte is surrounded by a diluent containing a polyanion such as IHP. After a time lapse of about 0.1 second, during which the outer membrane expands in an osmotic pulse, the cytoskeleton of the red blood cell becomes partially dissociated, expands with the outer membrane and remains attached to it. The cell is then permeable for a short period of time of about one second, and some of the haemoglobin is lost; however, recovery is rapid, and haemoglobin leakage stops after about one second. The cell remains mechanically fragile for several (e.g., about 5) more seconds. During this time it is believed that the cytoskeleton, which is still attached to the bilayer membrane, regains its original state.

30

An alternative osmotic pulse procedure is described in U.S. Patent No. 4,931,276 in which a polyanion is not employed. This procedure is summarised as follows:

5 Here, a polyanion is not added to the aqueous diluent medium in situations where the presence of a polyanion may be undesirable. Instead, a red blood cell is suspended in an aqueous diluent, such as a physiological buffer (e.g., phosphate buffered saline; PBS) which is neither an agent nor a co-factor, but is used in order to provide an essentially isotonic aqueous medium. As above, an osmotic pulse is applied relatively rapidly (e.g.,  
10 within about 0.1 second) and the outer lipid bilayer membrane swells. Since there is no polyanion present to dissociate the cytoskeleton, it is thought to remain intact and regain its original state relatively rapidly, if the amount of osmotic stress is not excessive. Where the osmotic stress, as determined by the concentration of the compound (e.g. DMSO) in the initial solution is high, some cells appear to go through a transitional form  
15 for several seconds, lose substantially all haemoglobin and become "ghosts". It is believed that the difference between groups of cells which regain their original state and those which go to the transitional form is whether or not the cytoskeletons remain attached to the bilayer outer membranes. Surprisingly, where the cells in the transitional form are subjected to shear stress at an appropriate time, they undergo resealing and  
20 regain substantially their original state. This has been confirmed experimentally by means of a capillary-induced shear stress.

The shear stress is applied following the osmotic pulse by passing red blood cells through a glass capillary tube of 2 x 0.01cm immediately after a brief delay time (seconds to  
25 minutes). The collected cell suspension is then centrifuged for 5 minutes at 1000 g and the supernatant is removed and saved. The packed cells are incubated for 30 minutes at 37°C to promote healing. After incubation, the cells are washed with PBS and samples are taken for measurement of recovered haemoglobin. In order to judge the number of viable cells present, the percent lysis is calculated from the recovered haemoglobin and  
30 the haemoglobin lost at the dilution and wash steps.

### iii. Chemical Crosslinking

A nucleic acid which is to be affixed to- or internalised by (e.g. by endocytosis or the migration of a receptor/ligand complex into the cytoplasm) a red blood cell in a method of the invention may be chemically crosslinked to cell surface structures, such as proteins, carbohydrates or lipids, or, for example, to a molecule which will bind a cell-surface structure, such as an antibody, ligand or receptor. Methods for chemical crosslinking are well known in the art, and numerous crosslinking agents with varying types and degrees of target specificity are available. Non-limiting examples of chemical crosslinking agents of use in the invention include: S-Acetylmercaptosuccinic anhydride; Adipic acid dihydrazide; 4-Azidobenzoic acid NHS ester; Bromoacetic acid NHS ester; Diethyl malonimide; Dimethyl pimelimide; 3,3-Dithiobis(propionic acid NHS ester) (Lomant's Reagent); Ethylene glycol bis(succinic acid NHS ester); Iodoacetic acid NHS ester; Polyoxyethylene bis(glycidyl ether); Polyoxyethylene bis(imidazolyl-carbonyl); and Suberic acid bis(NH ester).

### iv. Other Loading Techniques

Other methods which are well known in the art include, but are not limited to, standard cell transfection techniques, such as lipid- or cationic cell transfection, the use of a viral vector (e.g., a parvoviral vector) or inactivated virus particle, microinjection of cells and microparticle bombardment. The last of these entails coating gold particles with the nucleic acid to be loaded, dusting the particles onto a .22 calibre bullet, and firing the bullet into a restraining shield made of a bullet-proof material and having a hole smaller than the diameter of the bullet, such that the gold particles continue in motion toward cells in vitro and, upon contacting these cells, perforate them and deliver the payload to the cell cytoplasm. In addition, EP 0127925 describes the use of labelled leukocytes to detect the site of an infection. The method involves the ex vivo incubation of leukocytes with radioactively-labelled vesicles. The natural phagocytic properties of leukocytes is exploited to phagocytose the labelled vesicles, resulting in a pool of labelled leukocytes which are introduced into the patient. The patient is then scanned to identify a site of infection after the leukocytes have migrated to the site.

v. Combined Loading Techniques

As would be apparent to one of skill in the art, one or more of the above techniques can  
5 be used to load red blood cells for use in the invention, either simultaneously or in  
sequence. For example, U.S. Patent No. 4,224,313 discloses a process for preparing a  
mass of loaded cells suspended in a solution by increasing the permeability of the cell  
membranes by osmotic pressure or an electric field, or both, loading agents by passage  
10 from a solution through the membranes of increased permeability, restoring the original  
permeability by healing the membranes by regeneration effect, and separating the cells  
from the solution in which they were suspended. In that procedure, the agents in solution  
which are to be loaded include i) a pharmaceutical substance which reacts chemically or  
physically with substances in the extracellular milieu and which, when loaded into the  
15 cell, would prematurely destroy the cell membranes, and ii) at least one blood-compatible  
sugar and protein capable of providing hydrogen bridge bonding- or of entering into  
covalent bonds with the pharmaceutical substance, thereby inhibiting the reaction of the  
pharmaceutical substance with the cell membranes.

vi. Sequence of Loading of Nucleic Acids into Red Blood Cells

20 According to the invention, red blood cells may be co-loaded with two or more nucleic  
acids simultaneously or sequentially. If they are loaded simultaneously, they may be  
loaded by the same technique. If they are loaded sequentially, it is possible for one  
nucleic acid to be loaded, for example, by electroporation, while the next is loaded using  
25 an osmotic pulse. If chemical crosslinking to a surface structure is employed for one, but  
not both, of the nucleic acids, a different technique may be used to deliver the second  
nucleic acid to the RBC cytoplasm. In general, if loading is sequential, the nucleic acids  
can be loaded in any order. If loading is simultaneous, the two nucleic acids can be  
admixed prior to contact with the erythrocytes or can be added separately, prior to  
30 application of the stimulus which mediates uptake of the nucleic acids by the cell.

### C. Sensitising Loaded Cells in the Invention

It is necessary to apply an energy pulse (see below) to the target tissue with sufficient strength to disrupt loaded red blood cells; however, one must avoid damaging the target tissue or surrounding tissues. It is, therefore, necessary to render the cells administered according to the invention more fragile than the surrounding tissues (i.e., more susceptible to disruption by the energy pulse). It will be apparent to one skilled in the art that means to facilitate disruption (i.e., lysis) of cells exist in the art. These include, but are not limited to, a hematoporphyrin derivative (HPD) and Photofrin® (an adapted porphyrin), a photo-activatable agent which binds to cell membranes. In the absence of a source of activating photodynamic energy, Photofrin® is stable; however exposure to an activating source results in the selective lysis of Photofrin® coated cells and release of cellular contents. According to the invention, cells are coated with such a sensitising agent prior to introduction into the recipient mammal.

### D. Disrupting Red Blood Cell Vehicles of the Invention

An important feature of the invention is that it allows targeted delivery of nucleic acid to a tissue of interest within a mammal. According to prior art methods, such delivery requires that the nucleic acid to be delivered is carried to the target site by a vector (e.g., a viral vector) or other vehicle having biological affinity for a molecule specific to the target site. Such methods are limited by the availability of tissue-specific attractants for the vector or other vehicle, which may comprise a targeting molecule such as an antibody, receptor or ligand specific such a tissue-specific marker. The present invention provides a method of delivering a desired payload to a tissue of interest using non-targeted cells which are subjected to disruptive stress at the target site, thereby inducing release of the nucleic acid at the target site.

According to the invention, this is accomplished by directing a focused energy pulse at the target tissue as loaded red blood cells circulate through it. Electromagnetic radiation may be applied to the cells. This is particularly applicable to target tissues located on the surface of the subject mammal, although deep targets may also be exposed and irradiated.

Ideally, the electromagnetic radiation is in the form of light and is emitted by a laser. Methods employing laser energy to a subject mammal are widely used in the surgical and cosmetological arts, and appropriate instrumentation is well known in the art.

- 5 According to U.S. Patent No. 4,669,481, limited targeting of red blood cells to a small subset of mammalian tissues is achieved, if desired, as follows: Treating the RBCs under mild heating conditions will damage the cells, resulting in rapid sequestration by the reticuloendothelial system. The cells can be specifically targeted for the spleen by heating for 10 minutes at 49°C. Greater temperature or length of heating produces increased cell  
10 damage, with resultant hepatic uptake. Thus, if desired, payload deliver to the spleen or liver can be preferentially enhanced; however, the degree to which the payload is lost from damaged cells prior to administration is not known.

#### **E. Disease Treatment and Monitoring According to the Invention**

15

The invention is useful in the treatment of disease or injury by gene therapy as it provides a means of delivering a nucleic acid. Accuracy of targeting is assured through specific application of energy to the target site, tissue or cell within the patient.

#### **20 Dosage and Administration**

- Generally, nucleic acid molecules are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. When the end product (e.g. an antisense RNA molecule or ribozyme) is  
25 administered directly, the dosage to be administered is directly proportional to the amount needed per cell and the number of cells to be treated, with a correction factor for the efficiency of uptake of the molecules. In cases in which a gene must be expressed from the nucleic acid molecules, the strength of the associated transcriptional regulatory sequences also must be considered in calculating the number of nucleic acid molecules  
30 per target cell that will result in adequate levels of the encoded product. Suitable dosage ranges are on the order of, where a gene expression construct is administered, 0.5-1µg, or 1-10µg, or optionally 10-100 µg of nucleic acid in a single dose. It is conceivable that

dosages of up to 1mg may be advantageously used. Note that the number of molar equivalents per cell vary with the size of the construct, and that absolute amounts of DNA used should be adjusted accordingly to ensure adequate gene copy number when large constructs are loaded into a red blood cell vehicle of the invention and administered to a patient. As a first approximation, an amount of a nucleic acid molecule equivalent to between 1 and 10 copies thereof per cell should be delivered; one of skill in the art may adjust the ratio of nucleic acid molecules to cells at the target as is necessary to optimise nucleic acid uptake.

- 10 The precise amount of a nucleic acid required to be administered depends on the judgement of the practitioner and may be peculiar to each subject, within a limited range of values.

The number of loaded cells which are administered to a patient is in the range of  $1 \times 10^7$  to  $2 \times 10^{12}$  cells (at  $4-6.5 \times 10^9$  cells/ml). In order to calculate this number, the amount of a nucleic acid to be delivered to the target site, tissue or cell is determined. Red blood cells are loaded, and the efficiency of nucleic acid uptake is determined (e.g., by harvesting a fraction of the cells and determining empirically the amount of nucleic acid present per cell). The efficiency with which red blood cells are expected to reach the target is then taken into account. As a rough approximation, if the target site occupies 5% of the total body volume of the patient, approximately 5% of red blood cells circulate through the target at any given moment. The duration of the energy pulse to be used and the number of pulses are also taken into account. Lastly, the absolute body volume of the patient is measured. Given this information, it is within the knowledge of one of skill in the art to determine the particular number of cells which will deliver the needed dosage to the target site, tissue or cell.

Depending upon the needs of the patient, the nucleic acid may be released from loaded cells in a single dose or in multiple doses. If multiple doses are administered, repeated disruption of loaded cells administered in a single dose may be performed. Alternatively, and particularly if doses are spaced at long time intervals, loaded cells may be administered prior to each release of the nucleic acid. A typical mature red blood cell of a

human lives for about six weeks; when harvested for loading, cells of a given sample are from 1 to 42 days of age; therefore, three weeks after harvesting, half of the cells are dead. It is well within the knowledge of one of skill in the art to determine whether sufficient loaded cells remain in the patient at a given time after harvesting, loading and  
5 administration to yield a sufficient dosage of the nucleic acid.

#### F. Nucleic Acids of Use According to the Invention

A therapeutic nucleic acid of use in the invention may comprise a viral or non-viral DNA  
10 or RNA vector, where non-viral vectors include, but are not limited to, plasmids, linear nucleic acid molecules, artificial chromosomes and episomal vectors. Expression of heterologous genes has been observed after injection of plasmid DNA into muscle (Wolff J. A. et al., 1990, Science, 247: 1465-1468; Carson D.A. et al., US Patent No. 5,580,859), thyroid (Sykes et al., 1994, Human Gene Ther., 5: 837-844), melanoma (Vile et al., 1993,  
15 Cancer Res., 53: 962-967), skin (Hengge et al., 1995, Nature Genet., 10: 161-166), liver (Hickman et al., 1994, Human Gene Therapy, 5: 1477-1483) and after exposure of airway epithelium (Meyer et al., 1995, Gene Therapy, 2: 450-460).

One difficult aspect of providing a nucleic acid to a target cell is reproducibly obtaining  
20 high-level, tissue-specific, and long-term expression from genes transferred into the cell (for reviews see Mulligan, 1993, Science, 260: 926-932; Dillon, 1993, Trends Biotech., 11: 167-173). LCRs confer such position-independent activation on transgenes.

Locus Control Regions (LCRs) (Grosveld et al., 1987, Cell, 51: 975-985), also known as  
25 Dominant Activator Sequences, Locus Activating Regions or Dominant Control Regions, are responsible for conferring tissue specific, integration-site independent, copy number dependent expression on transgenes integrated into chromatin in host cells. The discovery and characterisation of LCRs are described in U.S. Patent No. 5,532,143, U.S. Patent No. 5,635,355, U.S. Patent No. 5,736,359, U.S. Patent No. 5,770,398 and U.S. Patent No.  
30 5,744,456, the complete disclosures of which are hereby incorporated by reference. First discovered in the human globin gene system, which was prone to strong position effects when integrated into the chromatin of transgenic mice or mouse erythro-leukemia (MEL)

cells (Magram et al., 1985, *Nature*, 315: 338-340; Townes et al., 1985, *EMBO J.*, 4: 1715-1723; Kollias et al., 1986, *Cell*, 46: 89-94; Antoniou et al., 1988, *EMBO J.*, 7: 377-384), LCRs have the ability to overcome such position effects when linked directly to transgenes (Grosveld et al., 1987, *supra*). Numerous LCRs have been defined in the art, including but not limited to the  $\beta$ -globin and CD2 LCRs (European Patent Application 0 332 667), the macrophage-specific lysozyme LCR (Bonifer et al., 1994, *Nucleic Acids Res.*, 22: 4202-4210), and a class II MHC LCR (Carson et al., 1993, *Nucleic Acids Res.*, 21: 2065-2072).

10 Therapeutic nucleic acid sequences useful according to the methods of the invention include those encoding receptors, enzymes, ligands, regulatory factors, and structural proteins. Therapeutic nucleic acid sequences also include sequences encoding nuclear proteins, cytoplasmic proteins, mitochondrial proteins, secreted proteins, plasmalemma-associated proteins, serum proteins, viral antigens, bacterial antigens, protozoal antigens and parasitic antigens. Therapeutic nucleic acid sequences useful according to the invention also include sequences encoding proteins, lipoproteins, glycoproteins, phosphoproteins and nucleic acids (e.g., RNAs such as ribozymes or antisense nucleic acids). Ribozymes of the hammerhead class are the smallest known, and lend themselves both to in vitro synthesis and delivery to cells (summarised by Sullivan, 1994, *J. Invest. Dermatol.*, 103: 85S-98S; Usman et al., 1996, *Curr. Opin. Struct. Biol.*, 6: 527-533). Proteins or polypeptides which can be expressed by nucleic acid molecules delivered according to the present invention include hormones, growth factors, neurotransmitters, enzymes, clotting factors, apolipoproteins, receptors, drugs, oncogenes, tumour antigens, tumour suppressers, structural proteins, viral antigens, parasitic antigens and bacterial antigens. The compounds which can be incorporated are only limited by the availability of the nucleic acid sequence encoding a given protein or polypeptide. One skilled in the art will readily recognise that as more proteins and polypeptides become identified, their corresponding genes can be cloned into the gene expression vector(s) of choice, administered to a tissue of a recipient patient or other mammal, and expressed in that

30 tissue.

### G. Kits for Nucleic Acid Delivery According to the Invention

A kit designed for the easy delivery of a nucleic acid to a recipient, whether in a research of clinical setting, is encompassed by the invention. A kit takes one of several forms, as follows:

I. A kit for the delivery of a particular nucleic acid to a subject comprises red blood cells and a nucleic acid. Alternatively, the red blood cells are supplied loaded with the nucleic acid for convenience of use by the purchaser. The cells are supplied sensitised for rapid use or, for greater stability, unsensitised. In the latter case, a sensitising agent such as HBP or Photofrin® is supplied with, but separately contained from, the cells. The cells of the kit are species-specific to the organism of interest, such as a primate, including a human, canine, rodent, pig or other, as desired; in other words, the cells are of like species with the intended recipient. The cells of the kit are, additionally, specific to the blood type of the intended recipient organism, as needed. Optionally, the kit comprises one or more buffers for cell sensitisation, washing, resuspension, dilution and/or administration to a mammal. Appropriate buffers are selected from the group that includes low ionic strength saline, physiological buffers such as PBS or Ringer's solution, cell culture medium and blood plasma or lymphatic fluid. The kit additionally comprises packaging materials (such as tubes, vials, bottles, or sealed bags or pouches) for each individual component and an outer packaging, such as a box, canister or cooler, which contains all of the components of the kit. The kit is preferably shipped refrigerated. Optionally, non-cellular components are supplied at room temperature or frozen, as needed to maintain their activity during storage and shipping. They may be in liquid or dry (i.e., powder) form.

II. A second kit of the invention comprises a nucleic acid and, optionally, a sensitising agent (e.g., HBP or Photofrin®) and buffers therefor (e.g., saline or other physiological salt buffer, culture medium, plasma or lymphatic fluid). In addition, the kit contains appropriate packaging materials, as described above. The individual components may be supplied in liquid or dry (i.e., powder) form, and may be at room temperature, refrigerated or frozen as needed to maintain their activity during storage and shipping.

Red blood cells for use with this kit are obtained independently (for example, they may be harvested from the intended recipient mammal).

## EXAMPLES

5

Experimental methods employed in Examples 1 through 6, below, may be summarised briefly, as follows:

### a. Preparation of Erythrocyte Suspensions

10

Unless otherwise stated human peripheral blood was harvested for experimentation. Samples of blood were collected in tubes (lithium heparin) and applied to a 2ml column (packed bed) containing  $\beta$ -cellulose/microcrystalline cellulose (1 ml of whole blood/2 ml column). Erythrocytes eluting from the column (1ml) were subsequently washed (2x) in  
15 saline by centrifugation (4,000 rpm in an Eppendorf centrifuge). Erythrocytes were suspended to yield a 1.5% hematocrit (Ht).

### b. Cell Loading

20 Electroporative loading was carried out by delivering single pulses to samples of erythrocytes suspended together with the relevant payload in 0.8 ml of 0.9% w/v saline. Each sample was placed in a 0.8 ml electroporation cuvette with an electrode gap of 0.4 cm. Cuvettes were connected to a BioRad Gene Pulser and the defined voltage and capacitance were preset prior to firing of the electric pulse. Unless otherwise stated,  
25 samples to be electroporated were placed on ice for 10 minutes prior to delivery of the pulse. In addition, samples were allowed to reseal for at least 1 hour post-delivery of the pulse.

### c. Sensitisation and Photoactivation

30

The sensitizer used in these studies was hematoporphyrin derivative (HPD) and was synthesised as described previously (Doughty, 1995, Methods in Porphyrin

Photosensitisation, pp. 313-323, Plenum Press, NY). A stock solution of 1mg/ml was prepared in saline. Photoactivation was achieved by exposing the relevant sensitised preparations to low energy laser radiation (which is that emitted by a 10 mW HeNe laser placed 17 cm above the samples) for the required period of time (approximately 5 minutes).

#### d. Plasmids

One of the plasmids used in this study as a marker for genetic functionality in *E. coli* was pAR2 (4.88 kb) and this was a derivative of the pANNA 1 clone of Winters et al. (1996, Biotechnol. Letts., 18: 1387-1390). The plasmid consists of the pUC18 vector comprising a fragment encoding a *Micromonospora chalcae*  $\beta$ -glucosidase, and this fragment is functionally expressed in *E. coli*. Subcloning of restriction fragments from pANNA1 yielded a 2.2 kb *AccI*-*EcoRI* fragment (with one internal *AccI* site) which exhibited a positive  $\beta$ -glucosidase phenotype (pNB1). This plasmid was again restricted with *AccI* and plasmid yielding a positive  $\beta$ -glucosidase phenotype in *E. coli* was obtained. This again contained the internal *AccI* site resulting from re-ligation, however, the orientation of the *AccI*-*AccI* fragment was not determined. As a result of this uncertainty, the plasmid isolated was named pAR2 and carried Amp<sup>+</sup> and  $\beta$ -glu<sup>+</sup> phenotypes. *E. coli* transformed with this plasmid could be detected by plating on ampicillin-containing L agar plates together with the chromogenic substrate, X-glu.

The other plasmid used in these studies was a commercially-available mammalian/*E. coli* shuttle system comprising the pSV- $\beta$ -galactosidase vector (Promega). This 6.8 kb vector comprises the LacZ coding region under the control of the SV40 promoter and enhancer and is used as a promoter control vector system to monitor transfection efficiencies in mammalian cells. In this vector, the *E. coli* gpt promoter located upstream from the LacZ gene and facilitates expression of the latter in *E. coli*. Transfected cells may be detected histologically using the chromogenic substrate X-gal.

Mini-preparation of plasmid DNA was carried out as described previously (Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, NY). Maxi-preparation of plasmid DNA was carried out using the Wizard® Plus MaxiPrep DNA purification system (Promega) according to the manufacturers instructions. Subsequent to preparation, DNA was quantified using a fluorimetric assay based on the use of the Hoechst reagent (bis-benzimide, H-33258, ICN Biomedicals, Inc.; USA).

Unless stated otherwise, techniques such as agarose gel electrophoresis, restriction endonuclease cleavage and DNA extraction (phenol/chloroform) were carried out as described by Sambrook et al., 1989, *supra*.

#### e. Tissue Culture

Chinese hamster ovary cells (CHO-K1) were obtained from the European Collection of Animal Cell Cultures (ECACC, Cat. No. 85051005; also available from the American Type Culture Collection, designation CCL 61) were maintained in Ham's F12 medium supplemented with 10% (v/v) foetal bovine serum at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Cells were subcultured and, where appropriate, prepared for transformation by treating monolayers with 0.05% (w/v) trypsin and 0.02% EDTA in PBS (8g NaCl, 0.2g KCl, 1.15g Na<sub>2</sub>HPO<sub>4</sub>, 0.2g KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) for 5 minutes at 37°C.

#### f. Transformation of CHO Cells

Aliquots of  $5.0 \times 10^6$  CHO cells suspended in PBS containing the relevant quantity of DNA were placed on ice for 10 minutes prior to electroporation. Cell suspensions were then aliquoted into electroporation cuvettes (electrode gap = 0.4 cm). Electric pulses (E-field strength = 1.875 kV/cm; capacitance 25µF, single pulse) were delivered to samples using the BioRad Gene Pulser apparatus. Samples were then placed on ice for a further 10 minutes prior to suspension in 12 ml of Ham's F12/FBS medium. Cells were plated into 2-ml wells on tissue-culture plates (Costar) and cultured for at least 24 hours before staining.

#### g. Detection of CHO Transformants

Medium was removed from each well to be stained and wells were rinsed with PBS. Cells were then fixed in 2% (w/v) formaldehyde and 0.2% (v/v) glutaraldehyde (0.5ml/well) for 5 min at 4°C. Fixative was removed and cells were washed in PBS and overlaid with the histochemical reagent (5mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub> and 1mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside [X-gal]). Plates were incubated at 37°C for at least 24 hours.

### 10 **EXAMPLE 1**

#### Energy-Stimulated Release of a Nucleic Acid from a Red Blood Cell (RBC)

##### Optimisation of cell loading parameters

15

In order to determine the optimal loading parameters for plasmid DNA into human erythrocytes, samples of peripheral blood were harvested and the erythrocytes were isolated, as described above. The hematocrit (Ht) was adjusted to 1.5% and 0.8 ml aliquots were mixed together with 100 µl amounts of pAR2 (571.90 µg/ml). Mixtures were placed in electroporation cuvettes (0.4 cm electrode gap), held on ice for 10 minutes and single electric pulses were delivered at 25µF, as described above. Following delivery of pulses, the mixtures were allowed to re-seal for 1 hour on ice and subsequently centrifuged. Supernatants were harvested and the DNA was extracted using phenol/chloroform and ethanol precipitation as described previously (Sambrook et al., 1989, supra). The DNA extracted and quantified; the amount of DNA present in the supernatant was subtracted from the starting total to determine the quantity of DNA associated with the erythrocytes (Fig. 1). Electric pulses were delivered at three voltage settings and optimal loading was achieved at 1.875 kV/cm. In addition to providing an optimal loading voltage, the results also indicated that as much as 56% of the pAR2 plasmid DNA remained associated with the erythrocytes within the voltage range examined at a capacitance of 25µF.

Test for Retention of Activity Following Release from an RBC

In order to determine whether release of pAR2 from sensitised human erythrocytes could be effected via light induction and whether DNA so released retains activity with respect to the  $\beta$ -glu+ phenotype, human erythrocytes were electro-loaded with pAR2 as above, sensitised and subsequently treated with low energy laser radiation. The DNA from supernatants (released DNA) and from the cell pellets (retained DNA) following centrifugation was extracted and used to transform *E. coli* in order to determine the functional integrity of the plasmid with respect to the  $\beta$ -glu+ phenotype, i.e., whether biological effector function was retained. The number of blue colonies when plated on agar containing ampicillin and the colorimetric substrate X-glu as a proportion of the total number of transformed colonies yielded a measure of the amount of damage to the plasmid during photoactivation using the laser. This experiment is briefly summarised as follows:

Erythrocytes were obtained and loading of DNA was carried out as above at 1.75 kV/cm at 25 $\mu$ F using 100 $\mu$ g aliquots of DNA in each cuvette. Loading was carried out using 2 equal samples, both retained on ice for 1 hour and subsequently washed twice by centrifugation in saline. The pellets were then suspended in 400 $\mu$ l of HPD (1mg/ml) in order to achieve sensitisation. Samples were then centrifuged and supernatants were decanted. Cells were washed twice by centrifugation in saline and finally the pellets were resuspended in 100 $\mu$ l of saline. Sample 1 provided the control, which was not irradiated, whereas Sample 2 was subjected to a 5-minute irradiation from a 10mW HeNe laser positioned 17cm from the surface of the sample. Following irradiation, both samples were protected from light and held at room temperature for 45 minutes. Samples were centrifuged, after which pelleted material and supernatants were harvested separately. DNA from the supernatants and cell pellets was extracted as described previously (Sambrook et al., 1989). Extracts were precipitated using ethanol, dried and redissolved in 10 $\mu$ l of TE buffer (Sambrook et al., 1989, supra). These extracts were then used to transform *E. coli* K12 C600 cells essentially as described previously (Sambrook et al., 1989, supra). Transformed cultures were plated onto agar containing ampicillin (100 $\mu$ g/ml), X-glu (50 $\mu$ g/ml) and IPTG (50 $\mu$ g/ml). Plates were incubated overnight at

37°C and the number of blue colonies which resulted from each transformation were counted. Table 1 represents a semi-quantitative analysis of transformation frequency achieved using plasmid DNA extracted from supernatants derived from irradiated and non-irradiated, photoactivated red blood cells.

5

Table 1

| Sample       | Treatment      | No. of Transformed Colonies Exhibiting $\beta$ -glucosidase phenotype* |     |
|--------------|----------------|------------------------------------------------------------------------|-----|
| Supernatants | Non-irradiated | 831                                                                    | 66  |
|              | Irradiated     | 1356                                                                   | 376 |

\* Results from two separate experiments

10

The maximum numbers of transformants were obtained from the supernatants derived from cells which were irradiated, demonstrating light-dependent release of DNA from the sensitised erythrocytes. In addition, the results established that the phenotypic functionality (biological effector function) of the DNA was retained, as indicated by a complete absence of white colonies on any of the plates. These results demonstrated that DNA was released from the sensitised erythrocytes in a light-dependent manner and that the functional integrity of the DNA, as reflected by its ability to confer the  $\beta$ -glu+ upon transformed cells, was retained following photodynamic activation.

#### 20 Analysis of Plasmid Isolated from Transformant Colonies Produced Using the DNA Photo-Released From the Sensitised Erythrocytes

The results above demonstrated that pAR2 was released from the sensitised erythrocytes using light and that the  $\beta$ -glucosidase gene was relatively intact following exposure to photodynamic activation. In order to further confirm this observation, plasmid DNA was purified from representative transformed colonies and subjected to restriction endonuclease cleavage site analysis.

25

Plasmid DNA was isolated from *E. coli* transformants which had been transformed with pAR2 prepared from irradiated and non-irradiated sensitised erythrocytes. This was cleaved with the restriction enzyme *AccI* and the resultant DNA fragments were electrophoresed on agarose gels (Figure 2). Samples of DNA released by light from the sensitised system are shown in lanes 1-5. Control samples isolated from colonies transformed with plasmid derived from sensitised erythrocytes which were not irradiated were electrophoresed and are shown in lanes 6-8. The expected pattern obtained from the controls as predicted from the original restriction endonuclease restriction site map of the vector would be a partial band of 4.88 kb consisting of the total linear construct of the pAR2 vector backbone and the full insert DNA, a band of 4.08 kb consisting of the vector backbone from which the *AccI*-*AccI* fragment had been deleted and a band of approximately 1 kb consisting of the deleted *AccI*-*AccI* fragment. In the three control samples, these diagnostic bands are present. In the samples derived from colonies which were transformed with DNA photo-released from the sensitised erythrocytes, the patterns are similar, apart from variations brought about by differential concentration of DNA and the presence of contaminants in DNA preparations derived from different colonies. The results support the data in Table 1 and suggest that DNA which undergone photo-release from sensitised erythrocytes remains unaltered.

20

#### Loading of a Mammalian Vector into a Red Blood Cell for Use in the Invention

As shown above, an *E. coli* expression/marker plasmid electro-loaded into human erythrocytes, released using light and subsequently used to transform *E. coli* retained biological effector function and structural integrity. As an extension and confirmation of those experiments and to increase the relevance of the work in terms of expanding the application range of the technology to include gene therapies, it was decided to examine the possibility of loading a mammalian control expression vector (pSV- $\beta$ -gal) into erythrocytes. Loading parameters employed were comparable to those used with plasmid pAR2, and the results are shown in Fig. 3. Pulses were delivered at three field strengths and the amount of DNA associated with the erythrocytes was determined as described for Fig. 1, above. Specific uptake of DNA was highest (30%) using an E-field strength of

30

1.875 kV/cm, which was somewhat lower uptake than was observed with pAR2. Significant uptake was also obtained at 2.75 kV/cm, although again this was lower than that observed for pAR2 at the same E-field strength.

5 Transformation of Mammalian Cells with pSV- $\beta$ -gal Photo-Released from Sensitised Human Erythrocytes

Since the mammalian vector system could be effectively loaded into erythrocytes it was of interest to determine whether or not the DNA could be released using light following  
10 sensitisation of the loaded system. As in the case with pAR2, it was also of interest to determine whether or not the released DNA could subsequently be employed to electro-transform a mammalian cell line with functional expression of the  $\beta$ -galactosidase marker gene. In this case erythrocytes (0.8ml; 1.5% Ht) were electro-loaded (1.875 kV/cm, 25 $\mu$ F) with pSV- $\beta$ -gal (approximately 100 $\mu$ g/0.8ml) and the system was sensitised by  
15 incubation with HPD (1mg/ml) for 1 hour at 2-5°C. Following washing by centrifugation the sensitised system was subjected to low energy laser radiation, as described above, for 5 minutes and subsequently retained at room temperature for 1.5 hours. A 0.4 ml aliquot of the resulting cell suspension was mixed with 0.4 ml of CHO cells (2.5 x 10<sup>6</sup> cells/ml) and the mixture was dispensed into an electroporation cuvette (0.4 cm electrode gap).  
20 After a 10-minute incubation on ice, a single pulse of 1.875 kV/cm at 25 $\mu$ F was delivered to the sample. The mixture was incubated on ice for 10 minutes and then resuspended in 12 ml of Ham's F12 medium supplemented with 10% (v/v) foetal bovine serum. Cells were plated at 2 ml per well and incubated for 72 hours prior to histological staining for  $\beta$ -galactosidase activity using the chromogenic substrate X-gal. Cells were fixed and  
25 stained as described above. Control samples consisted of erythrocytes loaded with pSV- $\beta$ -gal and treated in a similar manner to the sample above, except that sensitisation and irradiation steps were omitted from the procedure. The results obtained from this experiment are shown in Fig. 4A and Fig. 4B. Fig. 4A is a photograph representative of the cell population in the control sample, in which no transformants were visible, stained  
30 for  $\beta$ -galactosidase activity. Fig. 4B is a photograph representative of the cell population in the sensitised and photoactivated system; here, colonies exhibiting  $\beta$ -galactosidase activity were detected. In the photoactivated system, it was estimated that the

transformation efficiency was approximately 8-10% based on the area scanned by microscopy and the number of transformed foci detected. These results showed that the vector was released from the sensitised system using light and that the released DNA was biologically effective when electro-transformed into CHO cells. As was true with the  
5 bacterial expression vector used above, functional integrity of both the SV40 promoter/enhancer machinery and the  $\beta$ -galactosidase-encoding gene remained intact during photodynamic release from the red blood cell. It was interesting to note that the non-sensitised system yielded no transformants following electro-transformation. This suggests that the DNA associated with the erythrocytes is inaccessible for transformation  
10 unless the cells are pretreated so as to be receptive to the stimulus designed to release the biological effector 'payload' at the desired time and place, suggesting that an appropriate level of control is present for practice of the invention in a mammal.

In order to determine whether or not DNA would be taken up efficiently by CHO cells in  
15 the absence of an electro-transforming pulse, two red blood cell samples were loaded, sensitised and irradiated. Aliquots of 0.4 ml aliquots from both samples were mixed together with 0.4 ml of CHO cells ( $5 \times 10^6$  cells/ml). One cell mixture was subjected to an electro-transforming pulse, while pulse delivery to the other cell mixture was omitted. Mixtures were then suspended in 12 ml of medium and plated at 2 ml/well on a multi-  
20 well tissue culture plate. Cells from both samples were stained for  $\beta$ -galactosidase activity 72 hours after plating. In the absence of the electro-transforming pulse, no transformants were obtained following their disruption (Fig. 4C), which suggested that delivery of the pulse was a definite requirement for transformation. Cell mixtures subjected to the electro-transforming pulse did yield transformed foci (Fig. 4D) after  
25 disruption, which confirmed that it was necessary to apply the pulse to elicit transformation. The approximate transformation efficiency in the latter case was in the region of 10%.

**EXAMPLE 2**Co-loading of two plasmids into human erythrocytes

5 The objective of this experiment was to determine whether or not it would be possible to co-load two distinct plasmids, pAR2 and the mammalian promoter control vector pSV- $\beta$ -gal (Promega) into human erythrocytes using the electroporative technology. To this end peripheral human blood was harvested as described for Example 1 and suspended in saline to yield a 3% hematocrit. 0.8ml aliquots of cell suspensions were mixed together  
10 with 20 $\mu$ g of each plasmid in electroporation cuvettes (electrode gap =0.4cm) and pulses of 1.875kV/cm at 25 $\mu$ F were delivered to the samples using a BioRad Gene Pulser apparatus as described for Example 1. Cells were allowed to reseal for 1 hour and subsequently washed in saline. DNA was extracted from cell pellets using a Nucleo-spin blood kit (Bio/Gene, UK) and quantified by electrophoresis on agarose gels and  
15 subsequent scanning of the visualised bands using Phorenix 1D software loaded onto a PC. In order to determine extraction efficiencies DNA was incorporated added to intact cell pellets, cell lysates and to saline. On the basis of DNA extracted from these preparations it was determined that extraction efficiencies from cell lysates were approximately 43%. In order to confirm co-loading of both plasmids into the erythrocytes  
20 the DNA mixtures were analysed using agarose gel electrophoresis following digestion with the restriction endonuclease *Acc* I. Based on the restriction endonuclease cleavage site maps of both plasmids this would yield digests containing four DNA fragments; 2 from pAR2 consisting of approximately 1kb and 4.1kb and 2 from pSV- $\beta$ -gal consisting of 6.1kb and 0.7kb. The presence of all four bands in *Acc* I digests of the extracted DNA  
25 would confirm co-loading of both plasmids into the erythrocytes.

Cells were co-loaded with both plasmids as described and the loaded DNA was subsequently extracted. As a control both plasmids were incubated together with red blood cell lysates and the DNA was extracted in a similar manner in order to determine  
30 extraction efficiencies. The results obtained following extraction are summarised in Table 2 and they indicate that efficiencies of extraction were in the region of 40%. It was also found that 5.5 $\mu$ g of DNA was extracted from the loaded cells and this represented a

recovery of 14%. However on the basis of the determined extraction efficiency this would suggest that at least 11µg of DNA had been associated with the cells representing a loading efficiency of approximately 30%. In order to determine whether or not the extracted DNA consisted of a combination of both pAR2 and pSV-β-gal it was decided to  
5 digest the DNA with the restriction endonuclease *Acc* I and analyse the resulting DNA restriction fragments using agarose gel electrophoresis. The results obtained are shown in Fig. 5 which demonstrate that 4 bands of approximately 6.1, 4.1, 1 and 0.7kb were present. On the basis of the restriction endonuclease cleavage site maps of both plasmids the predicted sizes for each DNA fragment were present and this demonstrated that both  
10 plasmids were present in the DNA preparation isolated from the loaded human red blood cells.

TABLE 2

Extraction of plasmids pAR2 and pSV-β-gal from human erythrocytes co-loaded with  
15 both plasmids.

| SAMPLE*       | DNA EXTRACTED (µg) | % RECOVERY |
|---------------|--------------------|------------|
| 20 Control    | 16                 | 40         |
| DNA co-loaded | 5.5                | 14         |

\* In all cases 20µg of each plasmid was added to the sample. Control samples consisted  
25 of DNA added to cell lysates produced using the lysis buffer from the Nucleo-spin blood extraction kit.

**EXAMPLE 3**

Light-mediated release of pAR2 and pSV- $\beta$ -gal co-loaded into human erythrocytes which were subsequently photosensitised.

5

These experiments were designed to determine whether it was possible to achieve light dependant release of both plasmids which were co-loaded into human erythrocytes which were subsequently photosensitised. In addition it was of interest to determine whether or not the plasmids, released by the photodynamic lysis of the erythrocytes, would retain their biological function. In order to demonstrate the latter it was decided to transform *E. coli* with the extracted mixture and determine whether they retained the ability to confer their respective phenotypes on the recipient transformed strains. In the case of pAR2, recipient transformant strains should exhibit  $\beta$ -glucosidase activity and this could be detected using the colorimetric substrate bromochloroindolyl- $\beta$ -D-glucoside (X-glu).  
10 These strains would exhibit a blue colour when grown on plates containing the substrate. In the case of pSV- $\beta$ -gal, recipient transformed strains would exhibit  $\beta$ -galactosidase activity and this could be detected using the colorimetric substrate bromochloroindolyl- $\beta$ -galactoside (X-gal). This vector contains the *E. coli*  $\beta$ -galactosidase gene under the control of the *E. coli gpt* promoter and recipient *E. coli* strains are inoculated onto agar  
15 plates containing the colorimetric substrate they exhibit a blue colour. Human erythrocytes were prepared and loaded as described in Example 2. After resealing cells were washed twice in saline and then suspended in 400 $\mu$ l of HPD (1mg/ml). Sensitisation was carried out for 1 hour at room temperature and samples were again washed twice in saline. Cells were finally suspended in 100 $\mu$ l of saline and irradiated for  
20 5 min. using the HeNe laser as described for Example 1. Samples were allowed to rest at room temperature for 45min. and supernatants were harvested following centrifugation. Control samples consisted of cells co-loaded with both plasmids and sensitised except that exposure to irradiation was omitted. DNA from supernatants was extracted using the Nucleo-spin blood kit and these were quantified by electrophoretic analysis on agarose  
25 gels and subsequent gel scanning as described in Example 2. No detectable quantity of DNA existed in supernatants from the control non-irradiated samples whereas 1.45 $\mu$ g of DNA was recovered from the laser-treated samples. This accounted for 3.6% recovery of

30

DNA from the experiment although on the basis of extraction efficiencies the amount of DNA released during photodynamic lysis would be in the region of 8%. In order to determine whether or not the phenotypes of the plasmids were preserved during photodynamic release from the red blood cells it was decided to transform *E. coli* K12

5 C600 with the extracts from both the laser treated and non-treated control supernatants. On the basis of the quantitative data obtained with the sample extracted from the laser irradiated sample 3 $\mu$ l (representing 43.5ng of DNA in the laser-irradiated sample) aliquots from both the irradiated and non-irradiated samples were used in transformation mixtures. Following transformation, mixtures were inoculated onto separate plates

10 containing either X-gal or X-glu in order to detect production of  $\beta$ -galactosidase or  $\beta$ -glucosidase activity, respectively.

The plates were incubated at 37°C for 16 hours after which coloured colonies began to appear. The results obtained demonstrate that transformation mixtures contained colonies

15 exhibiting both phenotypes confirming that both plasmids co-existed in the transforming DNA mixture extracted from the cells. In addition the results demonstrated that the phenotypes encoded by each plasmid remained intact following photodynamic release from the erythrocytes. Counts obtained from the plates are shown in Table 3. The results confirm that a comparatively greater concentration of DNA existed in supernatants from

20 the laser-irradiated samples as indicated by the increased number of colonies on plates A and B. This result demonstrates light-mediated release of both plasmids from the co-loaded system.

TABLE 3

Colonies obtained following transformation with DNA released from co-loaded erythrocytes during photodynamic activation.

5

| TREATMENT  | Number of transformants/cm <sup>2</sup> |                      |
|------------|-----------------------------------------|----------------------|
|            | $\beta$ -galactosidase                  | $\beta$ -glucosidase |
| CONTROL    | 2                                       | 1                    |
| IRRADIATED | 73                                      | 40                   |

15

**EXAMPLE 4**

20 Controlled Delivery of a Nucleic Acid to a Target Site in a Mammal According to the Invention

In the above Examples, the construction of and release of payload molecules from loaded red blood cells was presented. The invention is, additionally, applicable in a multicellular organism (e.g., a mammal). Accordingly, red blood cells which are compatible with the recipient mammal in terms of species- and blood-group-specific antigens are obtained, optionally from the recipient mammal. The cells are loaded with a nucleic acid. Cells are then sensitised to disruption using HPD, as above. Loaded, sensitised cells are delivered intra-arterially to a recipient mammal. Useful numbers of cells are typically, but not exclusively, in the range of  $1 \times 10^7$  to  $2 \times 10^{12}$  cells. Prior to stimulation of the target tissue with a photodynamic energy source, such as a laser, which will disrupt the sensitised, loaded erythrocyte vehicle and deliver the payload to that tissue, sufficient time (at least 2 and, preferably, 5 minutes) is allowed to ensure that the cells so introduced have achieved

30

distribution throughout the body or have reached the target site. Alternatively, longer periods of time may elapse between delivery of cells to the recipient mammal and vehicle disruption, extending for as long as the lifetime of the vehicle; for a red blood cell of a human, this period is approximately 40 days.

5

A pulse of light is applied to a surface target site (e.g., an epithelial or endothelial tissue), at the energy level described above. Alternatively, the payload may be delivered to a 'deep' tissue, if such a tissue is directly exposed to the light source via the making of a large surgical incision or, if a smaller incision is made (e.g., approximately 1-2 cm in  
10 length), a probe, such as a fibre-optic light source, may be inserted in order to energise the tissue. The duration of an energy pulse is brief (milliseconds to seconds); alternatively, if a larger dose of the nucleic acid are to be delivered to the target site, the pulse is applied for a longer period of time (minutes, or even an hour) or a series of short pulses is applied, thereby disrupting larger numbers of loaded, red blood cell vehicles as fresh loaded cells  
15 continue to circulate through the target tissue. Using a microlaser, such as a molecular laser inserted at the target site, long exposure times become operable, as potential damage to non-vehicle cells at the target site is negligible. If desired, repeated doses of the nucleic acid are delivered to the target tissue, hourly, daily or weekly, as desired. Over time, assuming a single administration of loaded cells to the mammal, the dosage released with  
20 each energy pulse to the target tissue decreases in proportion to the number of loaded cells remaining in the mammal, following previous disruptions and natural attrition (i.e. through cell death) of the vehicle population.

25

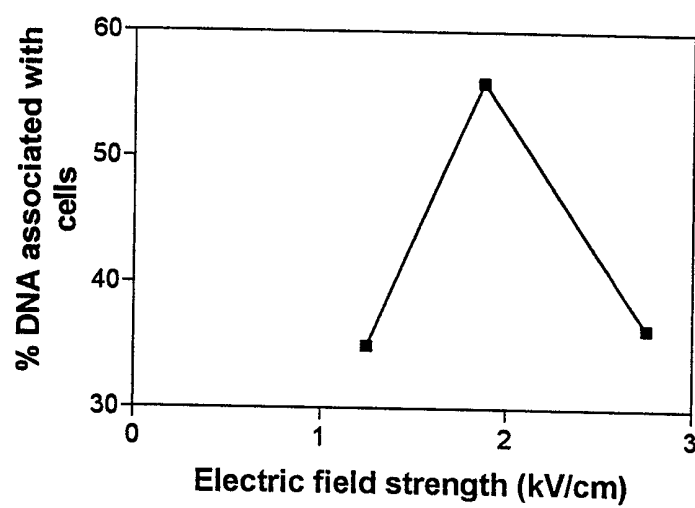
Release of the biological effector molecule at the target site is confirmed and its efficiency estimated by performing a detection step to detect the nucleic acid outside of red blood cells in the target tissue. Such monitoring may be effected by conventional nucleic acid detection and quantitation techniques.

## CLAIMS

1. A method for delivering a nucleic acid to a target site in an organism, comprising the steps of:
  - 5 a) loading a red blood cell with a nucleic acid;
  - b) introducing into the organism the red blood cell loaded with the nucleic acid; and
  - c) causing the nucleic acid to be released from the red blood cell at the target site in the organism.
- 10 2. A method according to claim 1, wherein the red blood cell is co-loaded with two or more separate nucleic acid molecules.
3. A method according to any preceding claim, wherein the red blood cell is contacted with a sensitising agent.
- 15 4. A method according to claim 3, wherein the red blood cell is contacted with the sensitising agent subsequent to the loading of the nucleic acid.
5. A method according to any preceding claim, wherein the nucleic acid is caused to  
20 be released from the red blood cell by disruption of the cell.
6. A method according to claim 5, wherein disruption of the red blood cell is performed by treatment of the cell with photodynamic energy.
- 25 7. A method according to any preceding claim wherein loading is performed by a procedure selected from the group consisting of electroporation, microinjection, membrane intercalation, microparticle bombardment, lipid-mediated transfection, viral infection, osmosis, osmotic pulsing, endocytosis and crosslinking to a red blood cell surface component.
- 30 8. A kit comprising a red blood cell, a nucleic acid and packaging materials therefor.

9. A kit according to claim 8 wherein the red blood cell is loaded with the nucleic acid.
10. A kit according to claim 8 or 9, further comprising a sensitising agent.
- 5
11. A kit according to any preceding claim, further comprising a liquid selected from the group consisting of a buffer, diluent or other excipient.
12. A kit according to claim 11, wherein the liquid is selected from the group
- 10 consisting of a saline buffer, a physiological buffer and plasma.
13. A method or kit according to any preceding claim, wherein the red blood cell is a human red blood cell.
- 15 14. A method according to any one of claims 1 to 7, wherein the organism is a mammal.

1/5

**FIG. 1**

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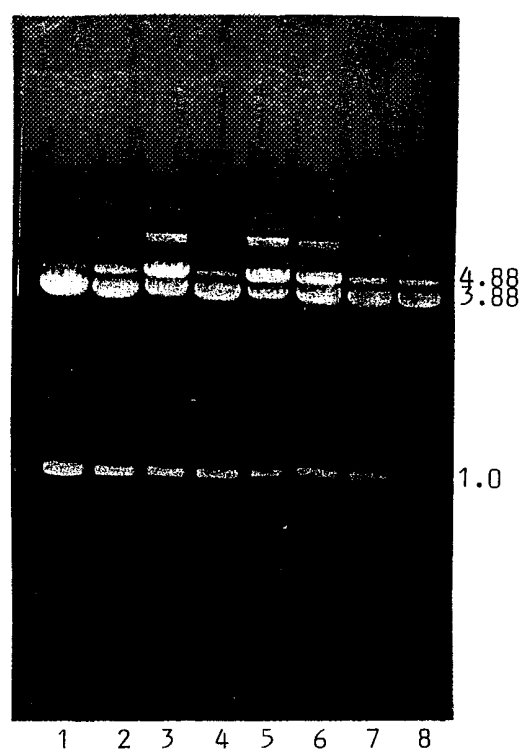


FIG. 2

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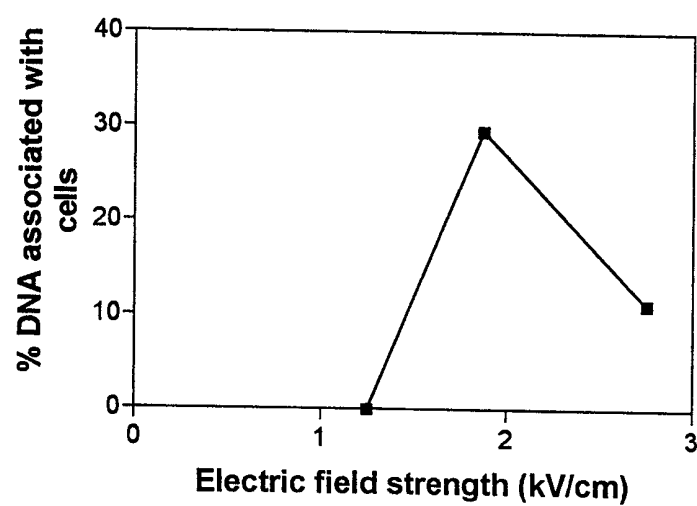
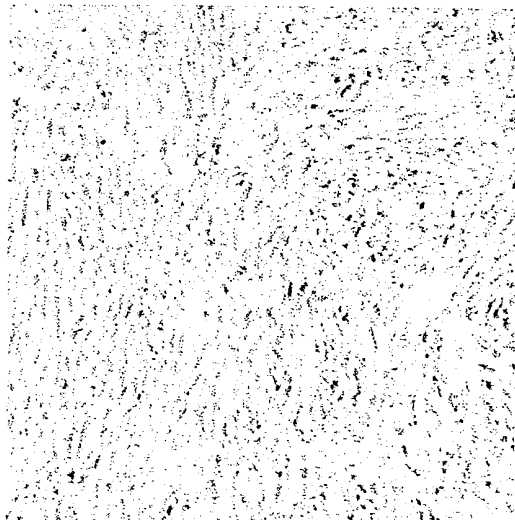


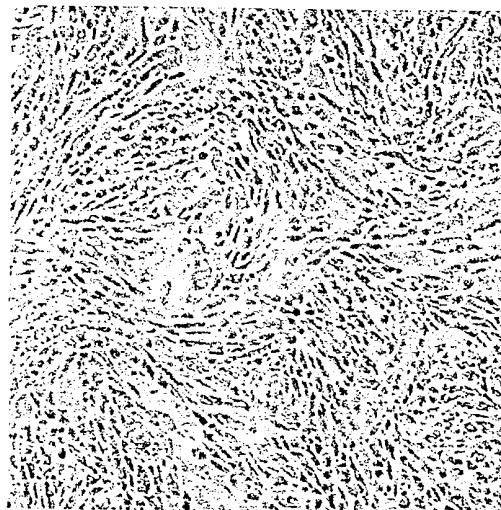
FIG. 3

4/5

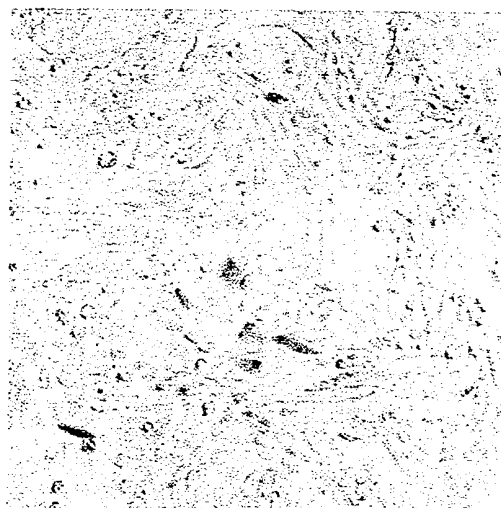
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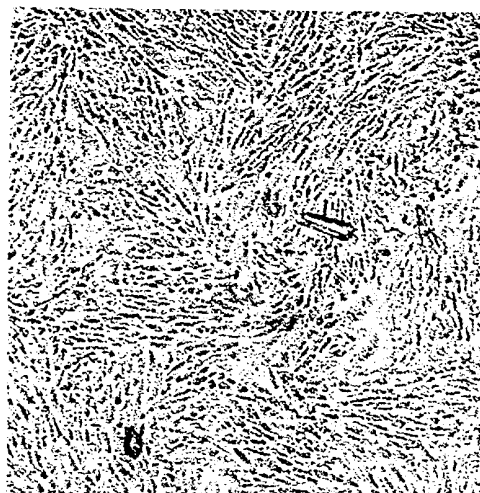
C



B



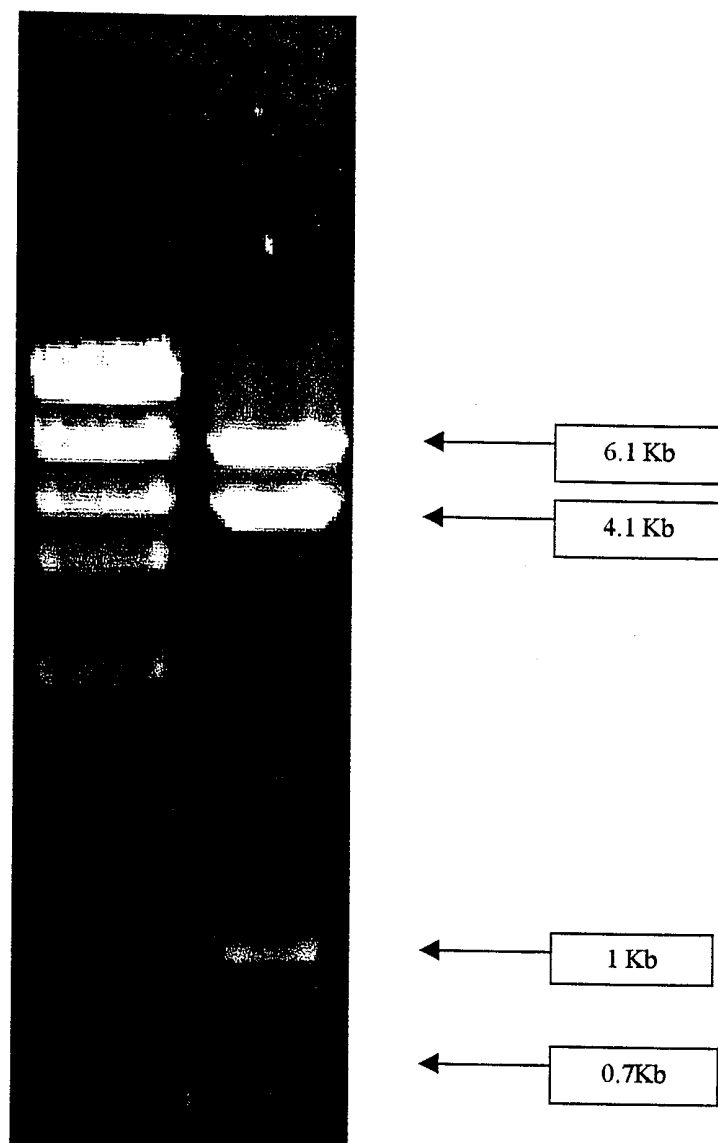
D



**FIG. 4**

1

2



**FIG. 5**

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/02490

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K48/00 A61K41/00 A61K47/00

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 7 A61K

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 practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                         | Relevant to claim No. |
|------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y          | FLYNN G ET AL: "Encapsulation of the thrombolytic enzyme, brinase, in photosensitized erythrocytes: a novel thrombolytic system based on photodynamic activation."<br>JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY. B, BIOLOGY, vol. 26, no. 2, November 1994 (1994-11), pages 193-6, XP000857148<br>page 196, left-hand column<br>page 194, left-hand column, line 23<br>-right-hand column, line 2<br>---- | 4-7                   |
| X          | US 5 653 999 A (GAUDREAU R ET AL)<br>5 August 1997 (1997-08-05)<br>column 4, line 62 -column 5, line 5<br>column 11, line 24 - line 32; claims 1-21<br>----                                                                                                                                                                                                                                                | 1-3,8-14              |
| Y          | -----<br>-/--                                                                                                                                                                                                                                                                                                                                                                                              | 4-7                   |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search

3 December 1999

Date of mailing of the international search report

15/12/1999

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Le Flao, K

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/02490

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                              | Relevant to claim No. |
|------------|-----------------------------------------------------------------------------------------------------------------|-----------------------|
| A          | WO 91 05041 A (TOWNES T ET AL)<br>18 April 1991 (1991-04-18)<br>claim 2<br>---                                  | 1-14                  |
| A          | US 4 931 276 A (FRANCO R ET AL)<br>5 June 1990 (1990-06-05)<br>column 1, line 16 - line 25; claims 1-9<br>----- | 1-14                  |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 99/02490

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 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:  
Remark: Although claims 1-7, 13 and 14 are partially directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
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.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 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 an additional fee, this Authority did not invite payment of any additional fee.
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.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/02490

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| US 5653999 A                              | 05-08-1997          | CA 2150617 A               | 08-08-1996          |
| WO 9105041 A                              | 18-04-1991          | AU 6505690 A               | 28-04-1991          |
|                                           |                     | US 5602306 A               | 11-02-1997          |
|                                           |                     | US 5766884 A               | 16-06-1998          |
| US 4931276 A                              | 05-06-1990          | NONE                       |                     |