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(54) Title: USE OF A BACTERIAL COMPONENT TO ENHANCE TARGETED DELIVERY OF POLYNUCLEOTIDES TO CELLS		
(57) Abstract An improved soluble molecular complex for targeting a polynucleotide to a specific cell is disclosed. The molecular complex comprises: (a) a polynucleotide; (b) a carrier made up of a polynucleotide binding agent and a cell-specific binding agent which binds to a surface molecule of the cell and is internalized into an endosome, and (c) a bacterial component or fragment thereof which lyses the endosome and causes the polynucleotide to be released into the cytoplasm of the cell. In a preferred embodiment of the invention the polynucleotide binding agent is polylysine, the cell-specific binding agent is an asialoglycoprotein, and the bacterial component is listeriolysin O. The disclosed soluble molecular complex and methods of use can be used therapeutically to deliver genes and antisense polynucleotides to specific cells <i>in vivo</i>.		

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USE OF A BACTERIAL COMPONENT TO ENHANCE TARGETED DELIVERY OF POLYNUCLEOTIDES TO CELLS

Government Support

5 The work that led to this invention was supported by one or more grants from the United States Government.

Background of the Invention

10 Rapid development in molecular biology has shown the potential of using gene therapy to correct hereditary disorders by the introduction of normal functioning genes into human tissue. Many techniques for gene delivery *in vitro* have been developed in the past few years. Among these are calcium phosphate precipitation (Gopal (1985) *Mol Cell Biol.* 5:1188; Graham et al. (1973) *Virology* 52:456), electroporation (Potter et al. (1984) *PNAS* 81:7161), microinjection (Harland et al. (1985) *Cell Biol.* 101:1094), liposomes
15 (Fraley et al. (1979) *PNAS* 76:3348) and retrovirus vectors (Guild et al. (1988) *Virology* 62:3795). However, intrinsic limitations have prevented some of these techniques from being widely used for *in vivo* gene delivery. For example, retrovirus vectors will only integrate into dividing cells and there is a size limit for the foreign gene that the vector can carry. In calcium phosphate precipitation, plasma membrane damage occurs.

20 Recently, a soluble DNA targeting system has been developed for delivering DNA specifically to hepatocytes and other cells (Wu et al. *J. Biol. Chem.* (1987) 262:4429; U.S. Patent No. 5,166,320). For delivery to hepatocytes, this system takes advantage of the receptor-mediated endocytosis of asialoglycoproteins by receptors highly selective for parenchymal liver cells. A soluble targetable conjugate has been developed consisting of a
25 cell surface receptor-binding entity, asialoorosomucoid (ASOR) and a DNA-binding entity, polylysine. Polylysine binds DNA in a strong, non-damaging electrostatic manner and protects the DNA from nuclease attack. This conjugate can be targeted specifically to asialoglycoprotein receptor- bearing cells, resulting in cellular uptake and expression of foreign genes. (U.S. Patent No: 5,166,320; Wilson et al. *J. Biol Chem.* (1992) 267:963; Wu
30 et al. *J. Biol. Chem.* (1989) 242:16985; Wu et al. *Biochemistry* (1988) 27(3):887; Wu et al. *J. Biol. Chem.* (1991) 266:14438).

 Several approaches have been reported to enhance expression of delivered genes. For example, Curiel et al. recently reported enhanced gene expression by
cotransfecting cells with intact adenovirus and a transferrin-polylysine-DNA complex (Curiel
35 et al. (1991) *PNAS* 88:8850). Wagner et al. included certain fusogenic peptides derived from the N-terminal sequence of the influenza virus hemagglutinin as part of a DNA complex, resulting in a substantial augmentation of the transferrin-polylysine-mediated gene transfer (Wagner et al. (1992) *PNAS* 89:7934).

Additional methods for enhancing expression of transfected genes would be beneficial.

Summary of the Invention

5 This invention pertains to an improved soluble molecular complex for targeting a polynucleotide to a specific cell for selective cellular internalization and to methods of using the molecular complex. The soluble molecular complex includes a polynucleotide complexed with a carrier component comprised of polynucleotide binding agent and a cell-specific binding agent which binds to a surface molecule of a cell and is
10 internalized into an endosome. The complex further includes a bacterial component or portion thereof which is capable of lysing an endosome resulting in release of the polynucleotide into the cytoplasm of the cell.

 The polynucleotide binding agent is a compound such as a polycation which stably complexes a polynucleotide under extracellular conditions and releases the
15 polynucleotide under intracellular conditions in functional form. To prepare a carrier, the polynucleotide binding agent is linked to a cell-specific binding agent which binds a cellular surface structure, typically a cellular surface receptor, which mediates internalization of bound ligands by endocytosis into cellular endosomes. The cell-specific binding agent can be a natural or synthetic ligand (e.g., a protein, polypeptide, glycoprotein, carbohydrate, etc.) or
20 it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex. A preferred target for the cell-specific binding agent is the asialoglycoprotein receptor of hepatocytes which binds to and mediates the internalization of galactose-terminal (asialo-) glycoproteins.

 To enhance delivery of a polynucleotide to a cell, a bacterial component or
25 active fragment thereof capable of lysing a cellular endosome is included in the molecular complex. Following internalization of the complex into an endosome of the cell, the bacterial component lyses the endosome resulting in release of the polynucleotide into the cytoplasm. Preferably, the bacterial component is pH dependent and is activated upon internalization into an endosome. Alternatively, the bacterial component is thiol-activated. A particularly
30 preferred bacterial component is listeriolysin O, or active component thereof.

 The soluble molecular complex of this invention can be used therapeutically to deliver polynucleotides, such as genes and antisense polynucleotides, to specific cells *in vivo*, *in vitro*, or *ex vivo*.

35 Detailed Description of the Invention

 This invention is based, at least in part, on the discovery that a bacterial component having the capacity to cause lysis of a cellular endosome can be used to enhance delivery of a polynucleotide to a specific cell. Accordingly, to enhance delivery of a polynucleotide into a cell via receptor mediated endocytosis, a bacterial component capable

of lysing an endosome is included in a soluble molecular complex. The term "bacterial component", as used herein, includes any part of a bacterium or any substance produced by a bacterium which has the ability to lyse a cellular endosome, including proteins and fragments or analogues thereof having endosomolytic activity.

5 A soluble molecular complex of the invention includes a polynucleotide noncovalently linked to a carrier comprised of a polynucleotide binding agent and a cell-specific binding agent. The molecular complex further includes a bacterial component or fragment thereof which lyses a cellular endosome. The bacterial component can be linked to a component of the carrier, such as a polynucleotide binding agent. Alternatively, the
10 bacterial component can be separately linked to a second polynucleotide binding agent and complexed with the remaining components of the molecular complex. As part of the molecular complex, the bacterial component or fragment thereof is internalized into the cell with the remaining components of the molecular complex (i.e., the polynucleotide, the polynucleotide binding agent, and the cell-specific binding agent) via the same endocytotic
15 pathway. The cointernalized bacterial component or active fragment thereof causes lysis of the endosome, thereby releasing the components of the complex into the cytoplasm of the cell, resulting in increased uptake and/or prolonged activity of functional polynucleotide in the cell.

 The polynucleotide to be delivered can be DNA, RNA or an analogue thereof.
20 For example, targeted polynucleotides can be genes encoding desired proteins, such as secretory proteins (see U.S. Patent Application Serial No. 710,558, filed on June 5, 1991, and Serial No. 893,736, filed on June 5, 1992), such as clotting factors and other blood proteins; cell surface proteins (see U.S. Patent Application Serial No. 695,598, filed on May 3, 1991), such as cell surface receptors for low density lipoproteins, for growth factors, or for
25 hormones; immunogenic proteins (see U.S. Patent Application Serial No. 699,891, filed on May 14, 1991, and Serial Number 882,669, filed on May 14, 1992), such as viral proteins (e.g., hepatitis B surface antigen or HIV envelope proteins) or protein of other pathogens.

 A polynucleotide encoding a desired protein is in a form suitable for expression by the target cell. For example, the polynucleotide can be a gene encoding
30 appropriate signal sequences which provide for trafficking to intracellular destinations or cellular secretion of the product. The signal sequence may be the natural sequence of the protein or an exogenous sequence. The gene is operably linked to appropriate genetic regulatory elements required for expression of the gene product by the target cell. Regulatory sequences are art-recognized and are selected to direct expression of the desired protein in an
35 appropriate cell. Accordingly, the term "regulatory sequence", as used herein, includes promoters, enhancers and other expression control elements. Such regulatory sequences are known and discussed in Goeddel, Gene expression Technology: Methods in Enzymology, p. 185, Academic Press, San Diego, CA (1990). The gene can be contained in an expression

vector such as a plasmid or a transposable genetic element along with the genetic regulatory elements necessary for expression of the gene and secretion of the gene-encoded product.

Alternatively, the targeted polynucleotide can be an antisense polynucleotide (see U.S. Patent Application Serial Number 07/941,368, filed on September 4, 1992, and
5 Serial Number 08/042,943, filed on April 5, 1993, and Serial Number 08/181,557, filed January 12, 1994). In other cases, the polynucleotide can be a gene encoding an RNA molecule which has catalytic activity, such as a ribozyme (see U.S. Patent Application Serial Number 08/222,615, filed on April 4, 1994). The molecular complex can contain more than one copy of the same polynucleotide or one or more different polynucleotides.

10 The carrier component of the complex is typically a conjugate of a cell-specific binding agent and a polynucleotide-binding agent. The cell-specific binding agent is an agent which targets the complex to a particular cell and which specifically binds a cellular surface structure which mediates its internalization into cellular endosomes by, for example, the process of endocytosis. The target surface structure for the binding agent can be a
15 protein, polypeptide, carbohydrate, lipid or combination thereof. Typically, the cellular surface structure is a surface receptor which mediates endocytosis of a ligand. Thus, the binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein, glycopeptide or glycolipid which has functional groups that are exposed sufficiently to be recognized by the cell surface structure. It can also
20 be a component of a biological organism such as a virus, or a cell (e.g., mammalian, bacterial, protozoan). The cell-specific ligand can also be an antibody, or an analogue of an antibody such as a single chain antibody, which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, galactose-terminal carbohydrates such as
25 carbohydrate trees obtained from natural glycoproteins, especially structures that either contain terminal galactose residues or can be enzymatically treated to expose terminal galactose residues, can be used. Alternatively, other ligands such as polypeptide hormones can be employed. In addition, naturally occurring plant carbohydrates such as arabinogalactan can be used as ligands. Other useful ligands for hepatocyte targeting include
30 glycoproteins having exposed terminal carbohydrate groups such as asialoglycoproteins (galactose-terminal). These galactose-terminal ligands can be formed by coupling galactose-terminal carbohydrates such as lactose or arabinogalactan to nongalactose-bearing proteins by reductive lactosamination. Examples of additional asialoglycoproteins include, but are not limited to, asialoorosomucoid, asialofetuin and desialylated vesicular stomatitis virus. Such
35 ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues.

For targeting the soluble molecular complex to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages (lymphoma), mannose 6-phosphate glycoproteins for fibroblasts (fibro-sarcoma), intrinsic factor-vitamin B12 and

bile acids (See Kramer *et al.* (1992) *J. Biol. Chem.* 267:18598- 18604) for enterocytes, insulin for fat cells, and transferrin for smooth muscle cells or other cells bearing transferrin receptors. Alternatively, the cell-specific binding agent can be a receptor or a receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by standard procedures.

The polynucleotide-binding agent of the carrier complexes the polynucleotide to be delivered. Complexation with the polynucleotide must be sufficiently stable (either *in vivo*, *ex vivo*, or *in vitro*) to prevent significant uncoupling of the polynucleotide extracellularly prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the polynucleotide is released in functional form within the cell.

In a preferred embodiment, the binding between the polynucleotide-binding agent and the polynucleotide is based on electrostatic attraction which provides sufficient extracellular stability, but is releasable intracellularly. Preferred polynucleotide-binding agents are polycations that bind negatively charged polynucleotides. These positively charged proteins can bind noncovalently with the polynucleotide to form a targetable molecular complex which is stable extracellularly but releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine (e.g., ranging from 3,800 to 60,000 daltons). Other noncovalent bonds that can be used to releasably link the expressible polynucleotide include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and streptavidin or avidin binding to polynucleotide-containing biotinylated nucleotides.

Preferred polynucleotide-binding agents are polycations. These positively charged materials can bind noncovalently with the negatively charged polynucleotide to form a soluble, targetable molecular complex which is stable extracellularly but releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine (e.g., ranging from 3,800 to 60,000 daltons). Other noncovalent bonds that can be used to releasably link the polynucleotide include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide antibodies bound to polynucleotide, and streptavidin or avidin binding polynucleotide containing biotinylated nucleotides.

To enhance delivery of a polynucleotide to a cell, a bacterial component or fragment thereof capable of lysing cellular endosomes is included in the molecular complex. The bacterial component or fragment is typically covalently linked, preferably by a disulfide bond, to at least one of the carrier components, such as a polynucleotide binding agent. Alternatively, the bacterial component or fragment is separately linked to a second

polynucleotide binding agent or a second cell-specific binding agent and complexed with the remaining components of the molecular complex.

In a preferred embodiment of the invention, the bacterial protein or peptide used in the molecular complex is activated upon internalization into a cellular endosome.

5 The term "activated", as used herein, means that the bacterial component or portion thereof is able to lyse the endosome (e.g., by undergoing a conformational change, causing it to bind to the endosomal membrane and lyse the endosome). For example, the activity of the bacterial component can be pH dependent, i.e., the bacterial component has endosomolytic activity at the acidic pH within an endosome, but not at the pH outside the endosome (i.e., in the cell
10 cytoplasm or outside the cell). Such a bacterial component is inactive extracellularly and will not harm a target cell or host when outside the cell or when released into the cytoplasm, but will become active after being exposed to the acidic environment inside an endosome of the cell.

In another preferred embodiment of the invention, the bacterial component is
15 thiol-activated. Thiol-activated bacterial components are produced by various gram positive bacteria, including, but not limited to, bacteria of the families *Bacillus*, *Streptococcus*, *Clostridium*, *Lactobacillus*, and *Listeria* (see e.g., Smyth, C.J. and Duncan, J.L., Bacterial Toxins and Cell Membranes, Chapter 5, p.129, Academic Press (1978); Alouf, J.E. and Geoffrey, C., Bacterial component Toxins, p.165, Academic Press (1984)). A thiol-activated
20 bacterial component is one which contains one or more sulfhydryl groups which, when reduced, activate the protein and, when oxidized, inactivate the protein. In one embodiment, a thiol-activated bacterial protein, such as LLO, or a fragment of the protein which has reduced immunogenicity as compared to the whole protein, is linked to a carrier via the protein's free sulfhydryl group(s), thereby preventing reduction of the sulfhydryl group(s) and
25 inhibiting activation of the protein. This configuration provides the advantage of preventing the bacterial protein or peptide from harming the target cell prior to internalization into an endosome of the cell. Once inside a cellular endosome, it is believed that the disulfide linkage between the bacterial protein and the carrier is reduced and the protein is released from the carrier in an active form, causing lysis of the endosome.

30 In a particularly preferred embodiment of the invention, the molecular complex includes the bacterial component Listeriolysin O (LLO), a 56 kD thiol-activated protein produced by *Listeria monocytogenes* or, even more preferably, a fragment of LLO which maintains endosomolytic activity yet has reduced immunogenicity, such as those described below in Example 3. LLO is fully active at pH 5.5, but loses most of its activity at
35 pH 7 (see e.g., Portnoy et al. (1992) *Infection & Immunity* 60: 2710). Therefore, as part of the molecular complex of the invention, it is believed that LLO is inactive extracellularly or in the cell cytoplasm at neutral pH and becomes active when the complex is internalized into the acidic environment of an endosome. Thus, LLO is particularly preferred for use in a molecular complex of the invention due to its lack of cytolytic activity ensuring little damage

to the membrane of the cell when LLO is contacted with the cell extracellularly. Following entry into an endosome,

it is believed that an active LLO binds to the membrane of the endosome and that such binding disrupts the permeability of the membrane, leading to lysis of the endosome.

5 Other pH dependent or thiol-activated bacterial components, such as pneumolysin, can also be included in the molecular complexes of the invention.

To produce a molecular complex of the invention, a carrier is formed by chemically coupling, preferably by a covalent interaction, a polynucleotide binding agent (e.g., polynucleotide) and a cell-specific binding agent (e.g., ASOR).

10 In one embodiment, the carrier is linked to a bacterial protein or fragment thereof capable of lysing on endosome.

In another embodiment, a conjugate of the bacterial component or fragment and the polynucleotide binding agent is prepared. Then, both that conjugate and the carrier are complexed with the polynucleotide. The resulting molecular complex includes a bacterial
15 component which is linked separately to a polynucleotide binding agent and noncovalently complexed with the remaining components of the complex (i.e., the carrier and the polynucleotide).

The carrier can be formed by chemically linking the cell-specific binding agent to the polynucleotide-binding agent. The chemical linkage is typically covalent. A
20 preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by Jung, G. et al. (1981) *Biochem. Biophys. Res. Commun.* 101: 599-606. Alternative linkages are disulfide bonds or strong noncovalent linkages as in avidin-biotin coupling.

The chemical linkage can be optimized for the particular cell-specific binding
25 agent and polynucleotide-binding agent used to form the carrier. Reaction conditions can be designed to maximize linkage formation but to minimize the formation of aggregates of the carrier components. The optimal ratio of cell-specific binding agent to polynucleotide-binding agent can be determined empirically. When polycations are used, the ratio of the components will vary with the size of the polycation and the size of the polynucleotide. In
30 general, this ratio ranges from about 10:1 to 1:1, preferably about 3:1 by weight. Uncoupled components and aggregates can be separated from the carrier by molecular sieve or ion exchange chromatography (e.g., AquaporeTM cation exchange, Rainin).

In one embodiment, asialoorosomucoid-polylysine conjugate is formed with the cross-linking agent 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide. After dialysis, the
35 conjugate is separated from unconjugated components by preparative acid-urea polyacrylamide gel electrophoresis (pH 4-5). The conjugate can be further purified on the carboxymethyl functionalized column (see U.S. Patent Application Serial No.08/043,008, filed April 5, 1993, the teachings of which are incorporated by reference herein).

According to one method for forming the molecular complex, the carrier and the bacterial component are linked. A preferred linkage is a disulfide bond. As described in the examples below, this linkage can be achieved using, for example a chemical cross linker such as N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP). Other cross-linking reagents which can be used include, for example, N-hydroxysuccinimidyl esters, N-Succinimidyl-(4-Iodoacetyl)aminobenzoate (SIAB), Sulfo-SIAB, Sulfo-succinimidyl-4-maleimidophenylbutyrate (Sulfo-SMPB), and water-soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (see e.g., Grabarek et al. (1990) *Anal. Biochem.* 185:131).

When using a thiol activated bacterial component, such as LLO, the carrier component can be thiolated using, for example, SPDP, and then linked directly to free sulfhydryl groups contained on LLO or peptides derived from LLO (see e.g., Cumber et al. (1985) *Meth Enzymol* 112:207). Linking LLO or peptides thereof to the carrier via its sulfhydryl groups can provide the advantage of ensuring that these thiol groups remain oxidized and that the activity of the LLO is inhibited while the complex is outside the target cell.

Following coupling of the carrier and a bacterial component, the resulting conjugate (carrier-bacterial component conjugate) is noncovalently complexed with a polynucleotide to via the polynucleotide binding agent. This can be achieved by a stepwise dialysis procedure. In one embodiment, for use with carriers made of polycations such as polylysine, the dialysis procedure begins with a 2 M NaCl dialyzate and ends with a 0.15 M solution. The gradually decreasing NaCl concentrations result in binding of the polynucleotide to the carrier-bacterial component conjugate. In some instances, particularly when concentrations of the polynucleotide and conjugate are low, dialysis may not be necessary; the polynucleotide and conjugate are simply mixed and incubated at room temperature for approximately 30 minutes.

The optimal ratio of polynucleotide to carrier-bacterial component conjugate for formation of a soluble molecular complex of the invention can range depending upon the type and size of the polynucleotide and the carrier-bacterial component conjugate used. For example, the molar ratio for oligonucleotides and carrier-protein conjugates made up of ASOR, polylysine, and LLO can range from about 1000:1 to about 10:1 (oligonucleotide to carrier-protein conjugate). The ratio can be determined for a particular polynucleotide and carrier-bacterial component conjugate by gel retardation assay as described in U.S. Patent No. 5,166,320, the teachings of which are incorporated by reference herein. In brief, the proportion of polynucleotide to carrier-bacterial component conjugate which completely retards the migration of DNA run on a polyacrylamide gel is taken as the optimal ratio for complex formation.

According to a second method for forming a molecular complex of the invention, a conjugate of a bacterial component or fragment thereof capable of lysing

endosomes and a polynucleotide binding agent or a cell specific binding agent (e.g., anti-cell surface receptor antibody) is formed. As an illustrative example, a bacterial component, such as LLO or a fragment of LLO having endosomolytic activity, is linked to a polynucleotide-binding agent (e.g. polynucleotide) using a chemical cross-linking reagents such as SIAB, Sulfo-SIAB, Sulfo-SMPB or EDC. Alternatively, the bacterial component or portion thereof can be coupled with the polynucleotide binding agent enzymatically through the action of an enzyme such as transglutaminase or biochemically such as through a biotin-streptavidin bridge. The resulting conjugate can then be separated from free bacterial component and free polynucleotide binding agent by molecular sieve or ion exchange chromatography (e.g., AquaporeTM cation exchange, Rainin) using standard techniques.

In a particularly preferred embodiment, a polynucleotide binding agent-bacterial component conjugate is produced with LLO or, even more preferably, a fragment of LLO having endosomolytic activity, and polylysine by linking free sulfhydryl groups or free carboxyl groups contained on LLO to the polylysine. For example, polylysine can be thiolated using SPDP and then coupled directly to free sulfhydryl groups on LLO or peptides of LLO. Coupling can be achieved by incubation at room temperature for approximately 18 hours.

Following formation of a polynucleotide binding agent-bacterial component conjugate, the conjugate is noncovalently complexed with the remaining components of the molecular complex (i.e., the carrier made up of a polynucleotide-binding agent and cell-specific binding agent and formed as described above) by incubation at room temperature. For optimal complex formation, the polynucleotide should first be mixed with the carrier in an amount which only partially saturates the negative charge of the polynucleotide (i.e., approximately one-fourth of the negative charges of the polynucleotide are neutralized by the polycation of the carrier). When using carriers made of polycations, such as polylysine, this ratio can be determined by standard gel retardation assay. After complexation of the carrier and polynucleotide, the polynucleotide binding agent-bacterial component conjugate is added to the reaction mixture to neutralize the remainder of the polynucleotide (see e.g., Wagner et al. (1992) PNAS 89 : 6099-6103).

The soluble molecular complex of the present invention can be used to selectively deliver polynucleotides to a target cell under a variety of conditions. For *in vitro* delivery of a polynucleotide, cultured cells can be incubated with the soluble molecular complex of the invention in an appropriate medium under conditions conducive to endocytotic uptake by the cells. The molecular complex can also be used *ex vivo* to enhance delivery of polynucleotides to cells or tissues which have been removed from an organism and will subsequently be returned to the organism.

For *in vivo* delivery of a polynucleotide, the molecular complex of the invention can be administered to an organism in a pharmaceutically acceptable vehicle. The term "pharmaceutically acceptable vehicle", as used herein, is intended to include any

physiologically acceptable vehicle for stabilizing the soluble molecular complex of the present invention for administration *in vivo*, including, for example, saline and aqueous buffer solutions. The use of such media for pharmaceutically active substances is well known in the art. Except insofar as any conventional media is incompatible with the molecular complex of the present invention, use thereof in a therapeutic composition is contemplated.

The soluble molecular complex can be administered parenterally. Preferably, it is injected intravenously. Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action or microorganisms such as bacteria and fungi.

This invention is illustrated further by the following examples which should not be construed as further limiting the subject invention. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

Example 1: Formation of a Soluble Molecular complex

A soluble soluble molecular complex for delivering plasmid DNA encoding the luciferase reporter gene from the firefly *Photinus pyralis* to cultured Huh-7 cells was produced as follows.

(a) Purification of Listeriolysin O (LLO)

LLO was purified according to the method of Geoffroy et al. (1987) *Infection & Immunity* 55:1641. In brief, *Listeria monocytogenes* strain 10403S (kindly provided by Dr. Portnoy from U. Pennsylvania) was propagated in iron-free media. Culture supernatant was concentrated via pressure ultrafiltration at 4°C using PM membrane (Amicon).

Thiolpropyl Sepharose 4B or 6B columns were used to eliminate proteins that had no sulfhydryl group. The final product was purified through a Toyopearl HW-50 gel filtration column. Products from each purification step were tested for hemolytic activity according to the method of Geoffroy et al. (*supra*) and protein concentration to determine the percentage of recovery. Purity of proteins from each step was checked by SDS-PAGE.

(b) Formation of the Carrier: Coupling Asialoorosomucoid (ASOR) With Polylysine (PL)

ASOR-PL carrier was formed by chemically linking ASOR, labeled with iodine-125, to PL via a peptide bond. The linkage was formed with a water soluble

carbodiimide as described by Jung, G. et al. (1981) *Biochem. Biophys. Res. Commun.* 101: 599-606. The carrier was purified by molecular sieve chromatography.

(c) Linking LLO to the Carrier

5 In order to couple LLO to the ASOR-PL carrier, the carrier was first thiolated by incubating at neutral pH for 30 minutes with SPDP [N-Succinimidyl 3-(2-pyridyldithio) propionate], a heterobifunctional cleavable crosslinker which reacts with primary amines contained on either ASOR or PL (Cumber et al *Meth Enzymol* (1985) 112:207). Briefly, to 10 mg of ASOR-PL in 1 ml of borate buffered saline, 25 μ l of a 20 mM solution of SPDP in 10 DMSO was added. Unconjugated SPDP was removed by a PD-10 column.

 Equal amounts of SPDP-ASOR-PL and LLO were then mixed to a total volume of 1 ml and reacted overnight at room temperature. The reaction mixture was then applied on a cation exchange HPLC system. The ASOR-PL-LLO conjugate was eluted with a continuous gradient of 20 mM sodium acetate (pH 5.1) to 20 mM sodium acetate plus 1 M 15 sodium chloride (pH 5.1) for 40 minutes. The conjugate was eluted at the twelfth minute. The total eluent was 8 ml.

 The amount of 2-pyridyl-disulfide introduced was determined by incubating the reaction product with dithiothreitol (DTT). The concentration of released pyridine-2-thione can be determined by measuring the absorbance at 343 nm (Molar extinction 20 coefficient at 343 nm = $8.08 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

(d) Forming the Soluble Molecular Complex: Noncovalently Complexing the Polynucleotide and the ASOR-Polylysine-LLO Conjugate

 To determine the optimal proportion of ASOR-polylysine-LLO conjugate to 25 DNA that yields a soluble complex, a gel retardation assay was performed. Briefly, increasing amounts of purified ASOR-polylysine-LLO conjugate were incubated in a 24 well dish for 30 minutes at room temperature with 2.0 μ g/well of plasmid DNA, pCMVL (TargeTech, Inc.), which contains the gene for luciferase (De Wet et al. (1987) *Mol. Cell Biol.* 7(2) 725-737) driven by the CMV promoter/enhancer. This allows DNA complex to 30 form in an electrostatic manner. Each sample was filtered through 0.45 μ membranes to ensure that the complexes to be used were soluble.

 Samples were then loaded on to an agarose gel for electrophoresis (see e.g., Wu et al. (1987) *J. Biol. Chem.* 262 : 4429-32). The proportion that completely retarded the migration of DNA in the gel was taken as the optimal ratio for complex formation.

35 Once the appropriate ratio of DNA to ASOR-PL-LLO had been determined, complexes were made up in large scale. Typically, 2 μ g of plasmid DNA (in 0.9 % saline) and 40 μ g of ASOR-PL-LLO conjugate (in 0.9% saline) were mixed to a total volume of 20 μ l and incubated at room temperature for 40 minutes to form the complex.

Example 2: *In Vitro* Transfection Assay Using the Soluble Molecular Complex

Soluble molecular complexes containing plasmid DNA, pCMVL (TargeTech, Inc.), were prepared according to the method described in Example 1. For transfection, complexes were then added to 500 µg of serum free Dulbecco's Modified Essential Medium (DMEM) with 2% calcium filtered through a 0.45 µ Durapore membrane (Milipore Inc.) and added to cultured Huh-7 (asialoglycoprotein receptor (+)) cells (Liang J. et al. (1993) *J. Clin. Invest.* 91:1241-1246). Control cells were transfected with ASOR-PL-pCMVL complex alone (prepared according to the method of Wu et al. (1988) *Biochemistry* 27(3):887) and with ASOR-PL-pCMVL complex and free Listeriolysin O.

The cells were incubated with the ASOR-PL-LLO-pCMVL complex for four hours at 37 °C, then the medium was removed and replaced with fresh DMEM containing 10% fetal bovine serum. The incubation was continued for forty-eight hours. Cell lysates were assayed in triplicate for luciferase activity in light units using a luciferase gene expression detection kit (Promega). The results are shown in Table 1 and demonstrate that complexes containing LLO produced a greater than 100-fold increase in gene expression over complex alone or complex and free LLO, without any evidence of toxicity to cells. Exposure of cells to complexes and free LLO only produced a four fold increase in gene expression.

Exposure of ASOR-PL-LLO-pCMVL complexes to SKHep-1 (asialoglycoprotein receptor (-)) cells produced no significant expression under any conditions (see Table 2). This demonstrates that the complexes are internalized into cells by binding to asialoglycoprotein surface receptors which are then internalized into cells via endocytosis.

Competition assays using a 100 fold molar excess of free radiolabeled ASOR were then performed to determine the specificity of the ASOR-PL-LLO-pCMVL complex. The results are also shown in Table 1. The free ASOR effectively competed with the ASOR-PL-LLO-pCMVL complex, resulting in a 3-log decrease in activity. This showed that the ASOR-PL-LLO-pCMVL complex is very target specific (i.e., is taken up by ASOR receptors only).

TABLE 1

Addition	Activity (Light Units/25 μ g Cell Protein)
ASOR-PL-DNA Complex	2,100 $\pm$ 1,180
ASOR-PL-DNA Complex + free LLO	9,780 $\pm$ 8,170
ASOR-PL-DNA-LLO Complex	223,440 $\pm$ 4,510
ASOR-PL-DNA-LLO Complex + excess AsOR	330 $\pm$ 9

5 TABLE 2

Addition	Activity (Light Units/25 μ g Cell Protein)	
	Huh-7 Cells	SKHep-1 Cells
ASOR-PL-DNA-LLO Complex	90,195 $\pm$ 6,991	107 $\pm$ 18

Example 3: Endosomolytic Peptides of LLO

In this example, peptides of listeriolysin O (LLO) having pH-dependent hemolytic activity were identified.

10 LLO protein is homologous to streptolysin O and pneumolysin in terms of the protein sequence. A peptide of 11 amino acids (ECTGLAWWWWR) (SEQ ID NO: 1) which are 100% conserved in these three proteins, contains a unique cysteine shown to be essential for the lytic activity (see. e.g. Mengaud et al. (1988) *Infection and Immunity* 56:766-772.) Therefore, to obtain peptides from LLO having lytic activity peptides containing this
 15 sequence were expressed and tested as follows.

A clone containing the entire coding region of LLO from a λ gt11 library of *Listeria monocytogenes* (purchased from Clontech) was isolated using oligonucleotides of known LLO sequences as hybridization probes. A 201 base pair fragment of this clone containing the putative active hemolytic domain (i.e., the domain encoding the amino acid
 20 sequence ECTGLAWWWWR (SEQ ID NO: 1) was then subcloned into the bacterial expression vector, pGEX-2X (obtained from Pharmacia Biotech). Purification of the resultant GST-LLO fusion protein was accomplished by binding of the GST tag to agarose beads containing glutathione. A thrombin protease site allowed subsequent cleavage of the GST domain from the LLO portion of the protein.

25 In order to test the expressed domain of LLO for lytic activity, hemolytic assays were performed on bacterial extracts containing the GST-LLO fusion protein and on the purified fusion protein as follows:

Blood (5 mls) was drawn into a purple tube (3.5% citrated tubes) and spun at 1000 rpm for 5 minutes. Serum/plasma was then removed from the sample and red blood cells (RBC) were washed two times with PBS, pH 7.0. An aliquot of 2 μ l was taken and cells were counted in a hemocytometer. The total number of cells for the 15 μ l RBC sample was measured to be approximately 10^8 RBC.

The 15 μ l RBC sample was then combined with 1 ml of PBS, pH 6.0 or 7.0. A 5 μ l sample of the bacterial extract or purified GST-LLO fusion protein was then added to 15 μ l of the RBC sample. A 5 μ l aliquot of the RBC sample was used as a negative control. A 15 μ l aliquot of the RBC sample, to which 1 ml of H₂O was added, was used as a positive control.

5 μ l of 1M DTT, a reducing agent, (final DTT = 5mM) was then added to the test samples and the samples were gently mixed by tapping the side of the tubes. Tubes were visually checked to ensure mixing of RBCs. Samples were incubated in a 37°C bath for 30-60 minutes, and then centrifuged at 1000 rpm for 5 minutes.

15 Samples were checked visually for cell lysis (red supt). Alternatively, optical density (OD) measurements could have been taken at A530nm.

Results

20 Bacterial extracts containing the GST-LLO fusion protein were positive for lysis of red blood cells. Purified fusion protein also had hemolytic activity, indicating that the activity was not due to some component in the bacterial extract. Extracts from bacteria producing only the GST portion of the fusion protein were negative for hemolytic activity, suggesting that the activity was due to the LLO portion of the fusion protein.

25 These results demonstrate that fragments of LLO having lytic activity (i.e., the ability to lyse cells or endosomes) can be recombinantly produced. These fragments of LLO can then be incorporated into the molecular complex of the invention, as described in Example 1, in place of the full-length LLO protein.

30

Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

35

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- 5 (i) APPLICANT: Wu et al.
- (ii) TITLE OF INVENTION: Use of a Bacterial Component to Enhance
10 Targeted Delivery of Polynucleotides to Cells
- (iii) NUMBER OF SEQUENCES: 1
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: LAHIVE & COCKFIELD
(B) STREET: 60 State Street, suite 510
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
20 (F) ZIP: 02109
- (v) COMPUTER READABLE FORM:
- 25 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII-text
- (vii) PRIOR APPLICATION DATA:
- 30 (A) APPLICATION NUMBER: 08/267,710
(B) FILING DATE: 06/29/94
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- 35 (A) NAME: Giulio A. DeConti, Jr.
(B) REGISTRATION NUMBER: 31,503
(C) REFERENCE/DOCKET NUMBER: TTI-107cp
- (ix) TELECOMMUNICATION INFORMATION:
- 40 (A) TELEPHONE: (617) 227-7400
(B) TELEFAX: (617) 227-5941
- (2) INFORMATION FOR SEQ ID NO:1:
- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: peptide
(C) Topology: linear
- 50 (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- 55 Glu Cys Thr Gly Leu Ala Trp Glu Trp Trp Arg
1 5 10

What is claimed is:

1. A soluble molecular complex for targeting a polynucleotide to a specific cell comprising:
 - 5 a) a polynucleotide;
 - b) a carrier comprised of a polynucleotide binding agent and a cell-specific binding agent which binds to a surface molecule of the cell and is internalized into an endosome; and
 - 10 c) a bacterial component which lyses the endosome resulting in release of the polynucleotide into the cytoplasm of the cell.
2. A soluble molecular complex of claim 1, wherein the polynucleotide is DNA.
- 15 3. A soluble molecular complex of claim 1, wherein the polynucleotide comprises a gene linked to appropriate genetic regulatory elements required for expression of the gene product by the target cell.
- 20 4. A soluble molecular complex of claim 1, wherein the polynucleotide is an antisense oligonucleotide.
5. A soluble molecular complex of claim 1, wherein the polynucleotide is a ribozyme directed against an RNA in the cell.
- 25 6. A soluble molecular complex of claim 1, wherein the polynucleotide binding agent is polylysine.
7. A soluble molecular complex of claim 1, wherein the cell-specific binding agent is an asialoglycoprotein.
- 30 8. A soluble molecular complex of claim 1, wherein the bacterial component is activated upon internalization into the endosome.
9. A soluble molecular complex of claim 1, wherein the bacterial component is pH dependent.
- 35 10. A soluble molecular complex of claim 1, wherein the bacterial component is thiol-activated.

11. A soluble molecular complex of claim 1, wherein the bacterial component is listeriolysin O.

12. A soluble molecular complex for targeting a polynucleotide to a specific cell comprising:

- a) a polynucleotide;
- b) a carrier comprised of polylysine and an asialoglycoprotein; and
- c) listeriolysin O.

13. A soluble molecular complex of claim 12, wherein the listeriolysin O is linked to the carrier by a disulfide bond.

14. A composition suitable for pharmaceutical administration comprising the molecular complex of claim 1 and a pharmaceutically acceptable carrier.

15. A method of delivering a polynucleotide to a specific cell comprising contacting the cell with a soluble molecular complex comprised of:

- a) a polynucleotide;
- b) a carrier comprised of a polynucleotide binding agent and a cell-specific binding agent which binds to a surface molecule of the cell and is internalized into an endosome of the cell; and
- c) a bacterial component which lyses the endosome resulting in release of the polynucleotide into the cytoplasm of the cell.

16. A method of claim 15, wherein the polynucleotide is DNA.

17. A method of claim 15, wherein the polynucleotide comprises a gene linked to appropriate genetic regulatory elements required for expression of the gene product by the target cell.

18. A method of claim 15, wherein the polynucleotide is an antisense oligonucleotide.

19. A method of claim 15, wherein the polynucleotide is a ribozyme directed against an RNA in the cell.

20. A method of claim 15, wherein the polynucleotide binding agent is polylysine.

21. A method of claim 15, wherein the ligand is asialoglycoprotein.
22. A method of claim 15, wherein the bacterial component is activated
5 upon internalization into the endosome.
23. A method of claim 15, wherein the bacterial component is pH dependent.
24. A method of claim 15, wherein the bacterial component is thiol-
10 activated.
25. A method of claim 15, wherein the bacterial component is
15 listeriolysin O.
26. A method of delivering a polynucleotide to a specific cell comprising
contacting the cell with a soluble molecular complex comprising:
- a) a polynucleotide;
 - b) a carrier comprised of polylysine and an asialoglycoprotein;
20 and
 - c) listeriolysin O.
27. A method of claim 26, wherein the listeriolysin O is linked to the
25 carrier by a disulfide bond.
28. A method of *in vivo* delivery of a polynucleotide to a specific cell of an
organism, comprising administering to the organism the composition of claim 14.
29. A soluble molecular complex for targeting a polynucleotide to a
30 specific cell comprising:
- a) a polynucleotide;
 - b) a carrier comprised of a polycation which noncovalently binds
the polynucleotide and a cell-specific binding agent; and
 - c) a fragment of a bacterial component having endosomolytic
35 activity.
30. The complex of claim 29, wherein the bacterial component is
listeriolysin O.

INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/US 95/08091

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/87 A61K47/48		
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 6 C12N 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	BIOTECHNIQUES, vol. 15, no. 6, 1993 pages 1018-1020, BARRY E.L.R. ET AL. 'introduction of antisense oligonucleotides into cells by permeabilization with streptolysin O' see the whole document ----	1-27,29, 30
Y	THE JOURNAL OF THE BIOLOGICAL CHEMISTRY, vol. 269, no. 17, 29 April 1994 pages 12918-12924, PLANCK C. ET AL. 'The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems' see the whole document ----- -/-	1-27,29, 30
☒ Further documents are listed in the continuation of box C. ☐ Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 27 October 1995		Date of mailing of the international search report 05.12.95.
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer Gurdjian, D

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/08091

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INFECTION AND IMMUNITY, vol. 55, no. 7, 1987 pages 1641-1646, GEOFFROY C. ET AL. 'Purification , characterization , and toxicity of the sulphydryl-activated hemolysin listeriolysin O from listeria monocytogenes' see the whole document ---	1-27,29, 30
P,X	GENE THERAPY, vol. 2, 1995 pages 498-503, GOTTSCHALK S. ET AL. 'Efficient gene delivery and expression in mammalian cells using DNA coupled with perfringolysin O' see the whole document -----	1-27,29, 30

INTERNATIONAL SEARCH REPORT

Int. application No.

PCT/US 95/08091

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 claim 28 is 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 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.