



- (72) CAMPOS, Manuel, US
(72) FRENCHICK, Patrick, US
(72) CLARK, Frederick, US
(72) OFFIT, Paul, US
(72) MOSER, Charlotte, US
(72) SPEAKER, Tully, US
(71) PFIZER INC., US
(71) TEMPLE UNIVERSITY OF THE COMMONWEALTH SYSTEM OF
HIGHER EDUCATION, US
(71) THE CHILDREN'S HOSPITAL OF PHILADELPHIA, US
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(54) **ENCAPSULATION DANS UNE BASE DE SOLVENT AQUEUX
D'UN VACCIN PURIFIE CONTRE LE VIRUS-HERPES BOVIN
DE TYPE 1**
(54) **AQUEOUS SOLVENT BASED ENCAPSULATION OF BOVINE
HERPES VIRUS TYPE-1 SUBUNIT VACCINE**

(57) Est décrite une substance purifiée microencapsulée provenant du virus-herpès bovin de type 1 (VHB-1). Sont également décrits des vaccins, des troussees et des méthodes servant à utiliser cette substance pour vacciner les bovins.

(57) A microencapsulated subunit component from bovine herpes virus-1 (BHV-1) is disclosed. Vaccines, kits, and methods for using the same to vaccinate a member of a bovine species are also disclosed.



ABSTRACT

A microencapsulated subunit component from bovine herpes virus-1 (BHV-1) is disclosed. Vaccines, kits, and methods for using the same to vaccinate a member of a bovine species are also disclosed.

AQUEOUS SOLVENT BASED ENCAPSULATION OF A BOVINE HERPES VIRUS TYPE-1 SUBUNIT VACCINE

Field Of The Invention

5 The present invention relates to microencapsulated vaccines. More particularly, the present invention relates to novel microcapsules having an anisotropic salt membrane encapsulating an aqueous or substantially aqueous core together with an immunogenic composition. The microcapsules are prepared by the interfacial reaction, in aqueous medium, of Lewis acid and base wall-forming reactants. More particularly, the present invention relates to a
10 subunit component of bovine herpes virus-1 (BHV-1) so encapsulated.

Background Of The Invention

 Microencapsulation is a process by which a relatively thin coating can be applied to dispersions of small particles of solids or droplets of liquids, thus providing a means for converting
15 liquids to solids, altering colloidal and surface properties, providing environmental protection, and controlling the release characteristics or availability of coated materials. Several of these properties can be attained by macropackaging techniques; however, the uniqueness of microencapsulation is the smallness of the coated particles and their subsequent use and adaptation to a wide variety of dosage forms and product applications. Heretofore, known feasible
20 methods for producing microcapsules on an industrial scale have often involved the use of organic solvents. However, the use of organic solvents may present environmental and safety problems. In addition, it is often difficult to remove all the organic solvent from the microcapsules, thus leaving organic contaminants.

 It has been proposed to use microcapsules as a means of delivering vaccine. Two broad
25 types of antigen delivery systems have been studied for their capacity to enhance immunity: solid (or porous) microcapsules and microcapsules with a core region surrounded by a physically distinct wall. Solid microcapsules may be prepared by a variety of processes including coacervation of colloids (Kwok, K.K., *et al.*, 1991, *Pharm. Res.* 8:341-344), precipitation of proteins by physical means (e.g., phase separation) (Santiago, N., *et al.*, 1993, *Pharm. Res.* 10:1243-1247) or chemical
30 agents (e.g., acid chlorides) (Levy, M.C., *et al.*, 1991, *J. Pharm. Sci.* 80:578-585.), or solvent evaporation techniques that surround aqueous dispersions with polyester films (Singh, M., *et al.*, 1991, *Pharm. Res.* 8:958-961). Wall/core systems shown useful for antigen delivery include liposomes (Gerlier, D., *et al.*, 1983, *J. Immunol.* 131:490), ISCOMS (Claassen, I., and Osterhaus, A., 1992, *Res. Immunol.* 143:531-541) and proteosomes (Gould-Fogerite, S., and Mannino, R.,
35 1992, *Liposome Technology*, Vol. III, Gregoriadis, G. (ed.), CRC Press, Boca Raton, FL.; Miller, M.D., *et al.*, 1992, *J. Exp. Med.* 176:1739-1744).

 Perhaps the best studied of the antigen delivery systems are those derived from the linear polymeric esters of lactic acid and glycolic acid (*i.e.*, poly (DL-lactide-co-glycolide)) (PLCG) (Edelman, R., *et al.*, 1993, *Vaccine* 11:155-158; Eldridge, J.H., *et al.*, 1989, *Curr. Top. Microbiol.*

Immunol. 146:59-66; Eldridge, J.H. *et al.*, 1990, *J. Controlled Release* 11:205-214; Eldridge, J.H., *et al.*, 1989, *Adv. Exp. Med. Biol.* 251:191-202; Eldridge, J.H., *et al.*, 1991, *Mol. Immunol.* 28:287-294; Eldridge, J.H., *et al.*, 1991, *Infect. Immun.* 59:2978-2986; Marx, P.A., *et al.*, 1993, *Science* 260:1323-1327; Moldoveanu, Z., *et al.*, 1993, *J. Infect. Dis.* 167:84-90; O'Hagan, D.T., *et al.*, 1993, *Vaccine* 11:149-154; O'Hagan, D.T., *et al.*, 1991, *Immunology* 73: 239-242; Ray, R., *et al.*, 1993, *J. Infect. Dis.* 167:752-755; Reid, R., *et al.*, 1993, *J. Immunol.* 150:323A; Reid, R.H., *et al.*, 1993, *Vaccine* 11:159-167). Encapsulation of putative antigens into PLCG microcapsules affords a number of advantages. First, microcapsules are easily degraded by hydrolysis to form lactic acid and glycolic acid. Second, PLCG microcapsules less than 5 μ m in size readily penetrate Peyer's patches, mesenteric lymph nodes and spleen after oral inoculation of mice. Third, oral, intraperitoneal, intranasal or subcutaneous inoculation of mice with PLCG microencapsulated antigens including influenza virus, parainfluenza virus, simian immunodeficiency virus, *Staph. aureus* enterotoxin B toxoid, and ovalbumin induces a greater immune response than that induced in animals inoculated with the same dose of free virus or protein. In addition, oral inoculation of mice with inactivated viruses induces an enhanced antigen-specific IgA response at mucosal surfaces. Lastly, PLCG microcapsules have been administered orally to adult volunteers without adverse effects.

The major disadvantage of PLCG microcapsules is the requisite use of organic solvents. Contact with organic solvents will inactivate the infectivity of viral and bacterial pathogens, and, in addition, may alter the immunogenicity of surface proteins critical to induction of humoral or cellular immune responses. In fact, large quantities of viral proteins have been required to induce an antigen-specific immune response with PLCG microcapsules.

Microencapsulation techniques are generally disclosed in U.S. Pat. 3,137,631; U.S. Pat. 4,205,060; U.S. Pat. 4,606,940; U.S. Pat. 3,959,457; and U.S. Pat. 5,132,117. In addition, microencapsulation techniques are taught in Belgium Pat. 882,476 to Lim (1980); U.S. Pat. 4,744,933; and U.K. Pat. Appl. 2 135 954 A to Dautzenberg *et al.* (1984). However, these techniques have drawbacks, including the use of organic solvents or heat, which can lead to inactivation or denaturation of the antigen. More specifically, most methods for preparing immunogens such as vaccines in encapsulated form (*e.g.*, PLGA's, cochleates, liposomes) require multiple, often harsh, processing steps (*e.g.*, introduction of surface active agents, generation of liquid/liquid interfaces with different surface energies, dispersion in reactive organic liquid phases, mechanical shearing during emulsification and/or vortexing, heating to remove one or another volatile components). Each of the several processing steps can exert an adverse influence on functional integrity of some or all of the initial quantity of immunogen used in the encapsulation. Such a reduction in functional integrity of some fraction of the initial charge of immunogen is manifested in reduced stability and immunogenicity of the resulting formulation, and counters the desired enhancement of immune response sought through encapsulation. With agents of low immunogenicity, the toll taken by multiple harsh processing steps may defeat any benefits to be derived by encapsulation using methods already known in the art.

-3-

By contrast, International Publication WO 95/28227 describes a microencapsulation technology that utilizes an all-aqueous system. This technology is based on the formation of poorly soluble (amine) salts of polyanionic macromolecules. Using this technology, immunogenic compositions are encapsulated using an entirely aqueous system of reagents at or below room temperature and without need for high pressures. This process is capable of producing uniform size particles under very gentle conditions, and may be used to
10 microencapsulate immunogenic compositions from infectious agents for use in vaccines.

An infectious agent of particular interest for encapsulation using the above-described all-aqueous system is bovine herpesvirus-1 (BHV-1), also known as infectious bovine rhinotracheitis virus. BHV-1 is a member of the alphaherpesviridae subfamily, and produces a variety of clinical forms of disease in cattle, including respiratory and genital infections, conjunctivitis, encephalitis, and abortions. Previous attempts at controlling BHV-1 infection
20 have utilized vaccines comprising live attenuated virus (Gerber, J.D., et al., 1978, Am. J. Vet. Res. 39:753-760; Mitchell, D., 1974, Can. Vet. Jour. 15:148-151), inactivated virus (Frerichs, G.N. et al., 1982, Vet Rec. 111:116-122), and viral subunits such as, e.g., one of the three major BHV-1 glycoproteins, which have been designated in the art as gI, gIII, and gIV (Babiuk, L.A., et al., 1987, Virology 159:57-66; van Drunen, S., et al., 1993, Vaccine 11:25-35). In addition,

-3a-

the ability of a recombinant, truncated version of the BHV-1 gIV glycoprotein (designated in the art as BHV-1 tgIV) to induce mucosal immunity against BHV-1 has been demonstrated (van Drunen, S., et al., 1994, Vaccine, 12:1295-1302). However, the immunoresponse to BHV-1 tgIV is low, requiring the administration of either large amounts of immunogen, which may be commercially impractical, or the addition of a potent adjuvant, which may in turn induce undesirable injection-site reactions. It was not known whether immunogenicity of a BHV-1 subunit component generally, and BHV-1 tgIV in particular, could be significantly enhanced by microencapsulation.

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Summary of the Invention

The present invention provides stable microcapsules that have aqueous cores and include immunogenic compositions comprising a subunit component of BHV-1. The microcapsules of the invention are produced according to methods based on those described in International Publication WO 95/28227. These microcapsules are substantially free of non-aqueous contaminants. The invention further provides a highly efficient method of preparing such microcapsules.

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In a first aspect, the present invention provides a microcapsule comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and an immunogenic composition surrounded by the salt membrane, said microcapsule being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of a selected water-

-3b-

soluble anionic polymer with a selected water-soluble amine, or the interfacial reaction of a water-soluble neutral salt of a selected anionic polymer with a water-soluble neutral salt of a selected amine. In a preferred embodiment, the selected anionic polymer

is alginic acid, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., sodium alginate), and the selected amine is spermine, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., spermine hydrochloride).

The immunogenic composition comprises a subunit component of BHV-1 which is capable
5 of inducing a protective immune response against BHV-1 when administered in the encapsulated form of the invention to a member of a bovine species. In a preferred embodiment, the subunit component of the immunogenic composition of the microcapsule comprises a BHV-1 glycoprotein such as, e.g., gI, gIII or gIV, or an immunogenic fragment or derivative thereof. In a more preferred
10 embodiment, the immunogenic composition of the microcapsule comprises a truncated BHV-1 glycoprotein, such as, e.g., BHV-1 tgIV (designated hereinafter as BHV-1-gD). In a more preferred embodiment, the immunogenic composition of the microcapsule is BHV-1-gD.

In a second aspect, the present invention provides a method of increasing the immunogenicity of a subunit component of BHV-1, comprising encapsulating said BHV-1 subunit component in a microcapsule comprising an anisotropic Lewis salt membrane encapsulating an
15 aqueous or substantially aqueous core, said microcapsule being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of a selected water-soluble anionic polymer with a selected water-soluble amine, or the interfacial reaction of a water-soluble neutral salt of a selected anionic polymer with a water-soluble neutral salt of a selected amine. In a preferred embodiment, the selected anionic polymer is alginic acid,
20 which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., sodium alginate), and the selected amine is spermine, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., spermine hydrochloride).

In a preferred embodiment, the BHV-1 subunit component comprises a BHV-1 glycoprotein such as, e.g., gI, gIII or gIV, or an immunogenic fragment or derivative thereof. In a more preferred
25 embodiment, the BHV-1 subunit component comprises a truncated BHV-1 glycoprotein, such as, e.g., BHV-1-gD. In a more preferred embodiment, the BHV-1 subunit component of the method is BHV-1-gD.

In a third aspect, the present invention provides a vaccine for protecting a member of a bovine species against BHV-1, comprising an immunogenic amount of the microcapsules of the
30 invention. The vaccine may further comprise a pharmaceutically or veterinarily acceptable carrier or diluent, and, optionally, an adjuvant.

In a fourth aspect, the present invention provides a method of vaccinating a member of a bovine species against BHV-1, comprising administering to the animal a vaccine comprising an immunogenic amount of the microcapsules of the invention.

35 In a fifth aspect, the present invention provides a kit for vaccinating a member of a bovine species against BHV-1, comprising a first container comprising an immunogenic amount of the microcapsules of the invention; and a second container comprising a pharmaceutically or veterinarily acceptable carrier or diluent. The microcapsules of the kit may optionally be supplied in lyophilized form. The kit may also optionally comprise an adjuvant.

-5-

In a sixth aspect, the present invention provides a combination vaccine for protecting a member of a bovine species against BHV-1 and, optionally, another disease or pathological condition, which combination vaccine comprises an immunogenic amount of the microcapsules of the invention; and an immunogenic amount of a second immunogenic composition capable of inducing a protective response against a disease or pathological condition in the member of the bovine species. The combination vaccine may further comprise a
10 pharmaceutically or veterinarily acceptable carrier or diluent, and optionally, an adjuvant.

The invention also comprises a commercial package comprising an immunologically effective number of microcapsules of the invention together with instructions for use in vaccinating a member of a bovine species against BHV-1.

Brief Description of The Figures

FIGURE 1 is the nucleotide sequence encoding BHV-1 glycoprotein IV (SEQ ID NO:1), and its deduced amino acid sequence (SEQ ID NO:2). BHV-1-gD (i.e., tgIV) extends from
20 amino acid residue 19 to residue 355.

FIGURE 2 demonstrates amine/polymer combinations which form insoluble salts according to the invention. A plus (+) mark indicates formation of a precipitate when reactants are combined. A zero (0) indicates that no precipitate forms.

FIGURE 3 demonstrates amine/polymer combinations which form stable microcapsules according to the invention. A plus (+) mark indicates that the reactants do form

-5a-

microcapsules. A zero (0) indicates that they do not.

FIGURE 4 is a schematic side view of an apparatus used in the preferred method for making the microcapsular material of the present invention.

Detailed Description Of The Invention

Applicants have surprisingly discovered that the immunogenicity of a BHV-1 subunit component is dramatically increased by microencapsulation. Specifically, Applicants have discovered that microencapsulation in spermine-alginate of a truncated form of the gIV glycoprotein of BHV-1 results in significantly increased immunogenicity compared to an unencapsulated control. Although microencapsulation has previously been shown to enhance the immunogenicity of other immunogens, no previous report reasonably predicted the observed degree of increase in immunogenicity of this BHV-1 subunit component resulting from encapsulation, as exemplified below.

Accordingly, the present invention provides a microcapsule, comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and an immunogenic composition surrounded by the salt membrane, said microcapsule being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of a selected water-soluble anionic polymer with a selected water-soluble amine, or the interfacial reaction of a water-soluble neutral salt of a selected anionic polymer with a water-soluble neutral salt

-5b-

of a selected amine, and said immunogenic composition comprising a subunit component of BHV-1 capable of inducing a protective response against BHV-1 when administered in the encapsulated form of the invention to a member of a bovine species.

The invention encompasses the use of a combination of any water-soluble anionic polymer with any water-soluble amine, or a combination of any water-soluble, neutral salt of an anionic polymer with any water-soluble, neutral salt of an amine, where the combination reacts to form stable microcapsules that can encapsulate and enhance the immunogenicity of the immunogenic composition of the invention.

In a preferred embodiment, the selected water-soluble anionic polymer is alginic acid and the selected water-soluble amine is spermine. Encapsulation of the immunogenic composition of the invention may be carried out by using alginic acid and spermine directly or, preferably, by using a corresponding water-soluble neutral salt of alginic acid and a corresponding water-soluble neutral salt of spermine. Such neutral salts of alginic acid and spermine are intended to include any neutral salts known in the art. In a preferred embodiment, encapsulation of the immunogenic composition of the invention is carried out by reacting the neutral sodium salt of alginic acid with the neutral hydrochloride salt of spermine.

In a further preferred embodiment, the subunit component of the immunogenic composition of the microcapsule comprises a BHV-1 glycoprotein such as, e.g., gI, gIII or gIV, or an immunogenic fragment or derivative thereof. In a more preferred embodiment, the immunogenic composition of the microcapsule comprises a truncated BHV-1 glycoprotein, such as, e.g., BHV-1-gD. In a more preferred embodiment, the immunogenic component of the microcapsule is BHV-1-gD.

The present invention further provides a method of increasing the immunogenicity of a subunit component of BHV-1, comprising encapsulating said BHV-1 subunit component in a microcapsule comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core, said microcapsule being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of a selected water-soluble anionic polymer with a selected water-soluble amine, or the interfacial reaction of a water-soluble neutral salt of a selected anionic polymer with a water-soluble neutral salt of a selected amine. In a preferred embodiment, the selected anionic polymer is alginic acid, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., sodium alginate), and the selected amine is spermine, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., spermine hydrochloride).

In a further preferred embodiment, the BHV-1 subunit component of the method comprises a BHV-1 glycoprotein such as, e.g., gI, gIII or gIV, or an immunogenic fragment or derivative thereof. In a more preferred embodiment, the BHV-1 subunit component comprises a truncated BHV-1 glycoprotein, such as, e.g., BHV-1-gD. In a more preferred embodiment, the BHV-1 subunit component of the method is BHV-1-gD.

The Immunogenic Composition:

As used herein, a "BHV-1 subunit component" refers to any structural component (peptide, protein, glycoprotein, polysaccharide, lipoprotein, etc.) comprising the capsid or envelope of BHV-

-7-

1, or any fragment or derivative thereof, or any nucleic acid polymer (DNA or RNA) encoding said structural component or fragment thereof, which is capable of directly or indirectly inducing a protective response against BHV-1 in a member of a bovine species when administered in encapsulated form according to the present invention. The BHV-1 subunit component may or may not be capable of inducing a protective immune response against BHV-1 when administered in an unencapsulated form to a member of a bovine species. The subunit component may be used in non-glycosylated form, or may be partially or fully glycosylated, or may have a different glycosylation pattern, compared to its native form.

BHV-1 may be isolated from tissues or organs of infected animals according to known techniques and propagated in susceptible cell cultures. See, e.g., van Drunen, 1994, supra. Alternatively, BHV-1 may be obtained from the American Type Culture Collection (ATCC). For example, the ATCC lists several BHV-1 strains, including strains having ATCC accession Nos. VR-631, VR-793, VR-2112, and VR-864, among others.

20 A variety of different subunit components of BHV-1, including their amino acid sequences and nucleotide sequences encoding them, are known in the art. For example, U.S. Pat. 5,151,267 to Babiuk et al. discloses the nucleotide sequences and deduced amino acid sequences of BHV-1 gI, gIII, and gIV. See also U.S. Pat, 5,585,264 to Babiuk et al. In addition, U.S. Pat. 5,545,523 to Batt et al. discloses BHV-1 specific oligonucleotides useful in the amplification of BHV-1 gI and

-7a-

gIV gene sequences. Furthermore, methods of purifying BHV-1 glycoproteins from virus-infected cell cultures have been described (Babiuk, L.A., et al., 1987 supra).

Van Drunen, et al., 1994, supra, describes the production of BHV-1 tgIV (i.e., BHV-1-gD), which is a truncated version of the gIV glycoprotein. BHV-1-gD was produced in transfected Madin Darby bovine kidney (MDBK) cells. MDBK cells are available from the ATCC (ATCC accession No. CCL22). The nucleotide sequence encoding the intact BHV-1 gIV glycoprotein (SEQ ID NO: 1) and its deduced amino acid sequence (SEQ ID NO: 2) are disclosed in U.S. Pat. 5,151,267 to Babiuk et al., and in Tikoo, S.K., et al., 1990, J. Virol. 64:5132-5142, and are presented herein in FIGURE 1. BHV-1-gD is a portion of BHV-1 gIV, and consists of 337 amino acids, beginning at amino acid residue No. 19 (Leu) of gIV, and terminating with amino acid residue No. 355 (Pro).

Production of BHV-1-gD or other BHV-1 subunit components for use in the claimed invention may be carried out according to recombinant techniques described in the above-cited references, or as described more generally in the art. See, for example, Maniatis, et al., 1989, Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel, et al., 1989, Current protocols in molecular biology, Greene Publishing Associates & Wiley Interscience, N.Y.; Sambrook, et al., 1989, Molecular cloning: a laboratory manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; and Innis et al. (eds) 1995, PCR strategies, Academic Press, Inc., San Diego.

The use of fragments and derivatives of BHV-1 subunit components are also within the scope of the invention where such fragments and derivatives are capable of inducing a protective response against BHV-1 when administered in encapsulated form according to the present invention. Such immunogenic fragments and derivatives can be produced by various methods known in the art. The manipulations which result in their production can occur either at the gene level or the protein level, or both. At the gene level, *e.g.*, a cloned DNA molecule encoding a BHV-1 subunit component can be modified *in vitro* by any of several known strategies. See, *e.g.*, Maniatis, *et al.*, 1989, *supra*; Ausubel, *et al.*, 1989, *supra*; and Sambrook, *et al.*, 1989, *supra*. Such modifications include, but are not limited to, endonuclease digestion, mutations that create or destroy translation, initiation, and/or termination sequences, or that create variations in the coding region, or any combination thereof. Any technique for mutagenesis known in the art can be used, including, but not limited to, exposure to a mutagenic agent, such as radiation or a chemical mutagen, or *in vitro* site-directed mutagenesis (see, *e.g.*, Hutchinson, *et al.*, 1978, *J. Biol. Chem.* 253:6551).

As a result of alterations at the gene level, the expressed polypeptide may contain changes, *e.g.*, deletions, additions or substitutions in amino acids, compared to the native molecule, which changes may result in one or more silent changes within the sequence to produce an immunogenic variant. For example, one or more amino acids of the native polypeptide can be conservatively substituted with an amino acid residue of similar charge, size or polarity, with the resulting polypeptide retaining its immunogenicity. Rules for making such substitutions are known in the art, and include, *e.g.*, those described by Dayhof, M.D., 1978, *Nat. Biomed. Res. Found.*, Washington, D.C., Vol. 5, Sup. 3.

Manipulation of the BHV-1 subunit component may also be made at the protein level. Any of numerous chemical modifications may be carried out by known techniques including, but not limited to: substitution of one or more L-amino acids of the native BHV-1 subunit component with corresponding D-amino acids, amino acid analogs, or amino acid mimics, so as to produce, *e.g.*, carbazates or tertiary centers; or specific chemical modification as, *e.g.*, with cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, or metabolic synthesis in the presence of tunicamycin, *etc.*

A BHV-1 subunit component, or a fragment thereof, may be derivatized by conjugation of one or more types of chemical groups thereto, including but not limited to acetyl groups, glycosyl groups, lipids, and phosphates, or by conjugation to other BHV-1 subunit components, other proteins, polyamino acids (*e.g.*, polylysine), polysaccharides, (*e.g.*, sepharose, agarose, or cellulose), serum albumins, keyhole limpet hemocyanin, or commercially activated BSA, among others, using known techniques. Such conjugation is preferably by covalent linkage at amino acid side chains and/or at the N-terminus or C-terminus of the polypeptide. Methods for carrying out such conjugation reactions are well-known in the field of protein chemistry.

Derivatives useful in practicing the claimed invention also include those in which a water-soluble polymer such as, *e.g.*, polyethylene glycol, is conjugated to the BHV-1 subunit component,

-9-

or to a fragment or derivative thereof, to provide additional desirable properties while retaining, at least in part, the immunogenicity of the encapsulated subunit component. These additional desirable properties include, e.g., increased solubility in aqueous solutions, increased stability in storage, increased resistance to proteolytic degradation, and increased in vivo half-life. Water-soluble polymers suitable for conjugation to the BHV-1 subunit component include, but are not limited to, polyethylene glycol homopolymers, 10 polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, polyoxyethylated polyols, etc. Methods of making water-soluble polymer conjugates of proteins are known in the art, and are described, among other places, in U.S. Pat. 4,179,337; U.S. Pat. 4,609,546; U.S. Pat. 4,261,973; U.S. Pat. 4,055,645; and U.S. Pat, 4,415,665.

Fragments and derivatives of the BHV-1 subunit component that are useful in practicing the claimed invention are those that are capable of inducing a protective response against BHV-1 in a member of a bovine species when 20 administered in encapsulated form according to the present invention. Such fragments and derivatives, once prepared, can be identified using nothing more than routine screening procedures known in the art. For example, a test fragment or derivative can be prepared and encapsulated as described herein, administered to a member of a bovine species, and the animal tested for seroconversion, e.g., by testing for the presence of virus-neutralizing antibodies in the serum, or for

-9a-

the ability of the vaccinated animal to resist subsequent challenge with BHV-1. Such methods for administering and screening are well-known in the art.

Microencapsulation Of The Immunogen:

The Lewis salt-walled aqueous-cored microcapsules of the present invention are prepared as described below. The capsular membrane is an ionically-stabilized, anisotropic Lewis salt membrane. The encapsulation system employs the essentially instantaneous reaction between immunogenic
10 composition-containing droplets of aqueous solutions of anionic polymers and low molecular weight cationic amine reactants, or between their water-soluble salts, to form water-insoluble films around the droplets and the immunogenic composition.

An aqueous solution or suspension of an immunogenic composition is dissolved or suspended in an aqueous solution of a suitable poly-anionic macromolecule (i.e., polymer), e.g., alginic acid. The resulting solution/suspension is then dispersed as droplets in an aqueous solution of a suitable
20 water-soluble amine, e.g., spermine. Alternatively, this reaction can be carried out using water-soluble, neutral salts of the anionic polymer and water-soluble, neutral salts of the amine. At the apparent interface of the polymer droplets and amine solution, a salt exchange reaction takes place that results in the formation of a very poorly soluble salt (formed between the amine and polymer) which precipitates to form more or less spherical beads or capsules in which the immunogenic

-9b-

composition is captured. The resulting suspension of microcapsules containing encased immunogenic composition is then collected.

The anionic polymer and reactant amine are chosen from groups which, on reaction with one another, will rapidly form a poorly soluble precipitate and so encase the droplets before the

polymer in the droplets diffuses sufficiently to distort appreciably the shape of the droplet or to lower the polymeric reactant concentration below that required to form a film. Thus, it is not necessary to employ polymer solutions of high viscosity, but it is necessary that the amine be capable of rapid diffusion to, and reaction at, the pseudophase boundary defined by the polymer droplet. The viscosity of the polymer solution may be as low as 2.5 to 10 centipoise.

A convenient means of dispersing the polymer solution droplets (comprising a solution or suspension of immunogenic composition in the polymer solution) in the amine solution is to allow an aerosol of the polymer solution to fall onto/into the amine solution as it is stirred. Use of a Bernoulli type nebulizer to generate the aerosol results in microcapsules with a relatively wide (Gaussian) distribution of particle sizes about the mean (with coefficients of variation approximating 10 to 20%). If narrower size distributions are desired, an acoustically-pulsed droplet generator as described herein may be used to provide highly uniform microcapsules (with coefficients of variability of diameter approximating 5%).

In some instances, as when acid labile immunogenic compositions are to be administered orally, it may be desirable to coat the microcapsules with an enteric material to protect them from gastric acid. Suitable enteric coating materials include cellulose acetate phthalate and polyoxyethylene crosslinked polymethacrylic acid. The technology for providing enteric coatings for small particles, tablets and capsules is well known in the pharmaceutical industry.

The reactants employed in preparing microcapsules from entirely aqueous solutions are available from a number of commercial vendors, but all described herein have been purchased from Fisher Scientific Company, F.M.C. Corporation, Ruger Chemical Company, Sigma Chemical Company, and/or The Upjohn Company.

Rapid release of encapsulated materials is accomplished by adding to an aqueous suspension of the microcapsules a water-soluble salt, either as a solid or as a solution of such salt. In either case, the salt employed must be capable of reacting with the insoluble film to yield water-soluble ionic products in a manner analogous to the reverse of the film-forming reaction. It is evident that the film-forming reaction is a reversible reaction. Slow release of soluble small molecules is realized through their gradual diffusion through the microcapsular walls. Diffusion rates depend on the size and solubility of the diffusing species and on the thickness and density of the capsular wall. Thus, in addition to providing a rapid release when wanted, the encapsulation process may be employed to provide controlled slow release of soluble encapsulated materials.

Anionic polymers or macromolecules which have been shown to be useful as encapsulating reagents are drawn from the group of water-soluble polymers with reactive carboxylate or sulfate groups consisting of alginic acids, alginic acids linked to fluorophores such as fluorescein isothiocyanate or rhodamine isothiocyanate, arabic acid, cellulose sulfate, carboxymethylcellulose, carrageenans, chondroitin sulfate, heparin, polyacrylic acid, polyoxyethylene crosslinked polyacrylic acid (e.g., EUDRAGIT L-100[®], produced by Rohm Pharma) and polyvinylcarboxylic acid (e.g., CARBOPOL 934[®]). In some preferred embodiments, the anionic polymer is selected from the group consisting of alginic acid (Fisher Scientific Co.,

-11-

Fairlawn, NJ), polyacrylic acid (Aldrich Chemical Co., St. Louis, MO), cellulose sulfate (Aldrich Chemical Co., St. Louis, MO), cellulose acetate phthalate (Eastman Organic Chemicals, Rochester, NY), Carbomer USP (CARBOPOL 934[®], B.F. Goodrich, Cleveland, OH), carboxymethylcellulose USP (medium viscosity, Ruger Chemical Co. Inc., Irvington, NJ), Heparin USP (The Upjohn Co., Kalamazoo, MI), and arabic acid (isolated according to the method described in U.S. Pat. 2,666,759, each of which is provided as a sodium salt. It is generally preferred that the anionic polymers be employed as their neutral salts with an alkali metal ion, e.g., sodium or potassium, or with ammonium or a trialkanolamine, e.g., triethanolamine, among others. In some preferred embodiments, the anionic polymer is alginic acid provided in its neutral salt form as sodium alginate.

Cationic reactants useful in preparing microcapsules according to this invention are drawn from the group of mono-, di-, tri- and tetra-amino compounds which includes: arginine, decylamine, dodecylamine, ethylenediamine, piperazine, methylene blue, octadecylamine, triethylamine, triethyltetramine, and spermine. In some preferred embodiments, the amine is selected from the group consisting of arginine (Sigma Chemical Co., St. Louis, MO), piperazine (Sigma Chemical Co., St. Louis, MO), ethylenediamine (Aldrich Chemical Co., St. Louis, MO), triethylamine (Aldrich Chemical Co., St. Louis, MO), triethylenetetraamine (Aldrich Chemical Co., St. Louis, MO), methylene blue (Fisher Scientific Co.,

-11a-

Fairlawn, N.J.), and spermine, each of which may be provided as a hydrochloride salt, and octadecylamine (Sigma Chemical Co., St. Louis, MO) which may be provided as an acetate. It is generally preferred that the amine is provided as a halide salt, e.g., chloride, bromide, or iodide, or as a low molecular weight alkanolic salt, e.g., having 6 carbon atoms or less, or that is soluble to at least 1% by weight in water, including e.g., an acetate, propionate or cyclopropionate salt, among others. In some preferred embodiments, the amine is spermine provided in its neutral salt form as spermine hydrochloride.

Prior to formation of microcapsules with captured immunogenic compositions, the anionic polymers and amines may be tested individually to determine their effect on immunogenicity of immunogenic composition. In order to determine the effect of anionic polymers and amines on immunogenicity, one having ordinary skill in the art can perform routine assays using readily available starting materials. For example, the ability of a selected immunogenic peptide or protein to invoke an immune response may be determined in the presence of various concentrations of the component being evaluated to ascertain the effect the component has on the immunogenicity of the peptide or protein.

Since in some embodiments it is preferred that the microencapsulated vaccine be effective when administered orally, the anionic polymers and amines which do not inactivate the immunogenic composition are tested in combination to determine their capacity to form microcapsules

-11b-

that resist breakdown in simulated gastric acid.

The aqueous sodium salt of anionic polymer, preferably 1 ml, is added dropwise to aqueous amine hydrochloride (or acetate), preferably 1 ml, to determine the capacity to form an interfacial precipitate. Combinations which generate solid material are used to make

microcapsules. Microcapsules are formed by dispersion of the sodium salt of anionic polymer as droplets approximately 5 μm in size into an aqueous solution of the amine salt in a manner analogous to that described for preparing calcium alginate microcapsules in U.S. Pat. 4,744,933, which is incorporated herein by reference. Short-term stability of microcapsules can be tested by observation at room temperature for 5 days in aqueous solution. Microcapsules stable at room temperature can be treated with simulated gastric acid (pH 1.2) at 37°C for 2 hours.

The combinations of anionic polymers and amines which do not inactivate the immunogenic composition, and which provide stable microcapsules, are then used to form microencapsulated vaccines. The immunogenic composition is first combined with the anionic polymer. The polymer/ immunogenic composition mixture is then dispersed as droplets into amine.

Microcapsules according to the present invention may be made with the apparatus described below, which allows preparation of microcapsules of extremely narrow size distribution at selectable median sizes. This is important in making microcapsules intended for injection, or for uptake through the gut-associated lymphatic tissues (a subset of which are often referred to as Peyer's patches) or the bronchus-associated lymphatic tissue of the respiratory system. Microcapsules intended for intravenous injection must necessarily be less than about 5 microns in diameter, small enough to pass through capillary beds. For administration by inhalation, particles must be in the respirable size range, *i.e.*, less than about 5 microns, while to reach deep alveolar sites it is preferred that particles be in the size range below 2 microns. The tissues of Peyer's patches are highly discriminatory in the size of particles which they will engulf, selecting only particles less than 10 microns and preferably about 5 microns in size. The device described below can produce populations of microcapsules of various sizes, with standard deviation from the mean of less than 0.25 micron in the range near the mean volume diameter of 5 microns.

Microcapsule Forming Reactions:

In accordance with the present invention, one of two types of reactions can be employed to form the microcapsules in aqueous media. These are acid-base reactions and salt exchange reactions.

5

Acid-base Reaction:

Several water-soluble acidic polymers will react in aqueous solution with low molecular weight water-soluble mono- or oligo-amines to form poorly water-soluble salts which may precipitate. The group of water-soluble acidic polymers which participate in this reaction includes arabic acid, cellulose sulfate, chondroitin sulfate, heparin, and fluorescent derivatives of the above
10 acidic polymers. The group of amines which yield poorly water-soluble salts in this reaction includes arginine, decylamine, dodecylamine, ethylenediamine, hexadecylamine, methylene blue, octadecylamine, piperazine, spermine, tetradecylamine, triethylamine and triethylenetetramine.

15

Salt Exchange Reaction:

A similar reaction occurs with formation of poorly water-soluble salts if the acidic polymers described above or certain relatively poorly water-soluble acidic polymers are employed as solutions of their respective neutral salts (e.g., sodium, potassium, ammonium or triethanolamine, among others), and the amines described above are dissolved as their water-soluble salts (e.g.,
20 hydrochloride, hydrobromide, acetate, or propionate, among others). The similar reaction may be considered a salt exchange reaction in which one of the products (e.g., sodium chloride or sodium acetate) is soluble and the amine-polymer salt is poorly soluble. The group of acidic polymers useful as their water-soluble salts in the salt-exchange reaction includes those polymers named above and also the following acidic polymers in the form of their sodium or other water-soluble
25 salts: alginic acid and fluorescent derivatives, e.g., fluorescein isothiocyanate and rhodamine isothiocyanate, derivatives of alginic and other acids, carboxymethylcellulose, cellulose acetate phthalate, EUDRAGIT L-100[®] (polyoxyethylene crosslinked polyacrylic acid), polyacrylic acid, polyvinylacrylic acid and fluorescent derivatives of these polymers. This larger group of acidic polymers will all react as their sodium or other water-soluble salt with at least one of the members
30 of the group of amines in the form of their hydrochloride or acetate salts to form poorly water-soluble amine-polymer salts in the salt exchange reaction. However, not all combinations that form poorly soluble salts will form microcapsules in accordance with this invention. See the test described below and compare FIGURES 2 and 3.

35

As used herein, the terms "anionic polymer", "polymer strand" and "anionic polymer solution" are meant to refer to polymers which participate in forming the amine-polymer salt. References to concurrently formed water-soluble products of the exchange reaction (e.g., sodium chloride or sodium acetate) are not expressly referred to herein unless there is specific need to refer to these water-soluble products.

Thus, the group of anionic polymers that can be used as microcapsule-forming agents in accordance with this invention consists of polymeric substances with carboxylate acidic functional groups (alginic, arabic, carboxymethylcellulose, EUDRAGIT L-100[®], polyacrylic acid and polyvinylcarboxylic acid), sulfate acidic groups (carrageenans, cellulose sulfate, chondroitin sulfate, heparin), linear or branched polyalkylene backbones (polyacrylic acid, poly-vinylcarboxylic acid), linear carbohydrate backbones (alginic acid, cellulose sulfate, chondroitin sulfate, heparin) and branched carbohydrate backbones (arabic acid).

In the microcapsules of the present invention, the anionic polymer constitutes the major structural component of the microcapsular walls. Typically, the polymer is chosen to provide a desired range of spacing between nearest neighbor anionic groups. Thus, in their extended forms above the theta temperatures of the polymers, the interanionic distances approximate the equivalent of 2 methylene groups in poly-acrylic acid, 6 in alginic acid, cellulose sulfate, chondroitin sulfate, and heparin, 10 in carboxymethylcellulose and between 20 and 30 in highly branched arabic acid. This allows one to selectively form capsule walls having different porosities. See U.S. Pat. 3,959,457 to Speaker *et al.* (esp. col. 5, l. 6-20).

Because all the anionic polymers used in the present invention have average molecular weights well above 10 kD, they are multivalent and can react with the amines in a wide range of stoichiometries. In practice it has been found that the preferred range of stoichiometries of amine to anionic polymer repeat unit is about 0.2 to about 0.6. In other words, about 2 to 6 amine molecules are available to combine with each 10 anionic groups on the polymers, to form salts of the polymer. Furthermore, because several of the amines are also multivalent, the reacting species can, in theory, form complex networks in which the amines serve to crosslink anionic polymer strands. The precipitates, which form essentially instantaneously when solutions of anionic polymers and amines are stirred together, tend to be amorphous, cohesive, adhesive and often filamentous. However, not all combinations of anionic polymer and amine yield poorly soluble amine-polymer salt. FIGURE 2 lists two groups of reactive species tested to date and indicates the combinations which have been successful in forming precipitates when these combinations were allowed to react. The anionic polymers and amines are listed in order of increasing approximate equivalent weights (shown in parentheses) of the amines and polymer repeating units.

Although the majority of the combinations of anionic polymer and amine will react to form a poorly water-soluble amine polymer salt, a smaller subset of this set appears to be capable of forming microcapsules, at least under the conditions tested to date. Thus, the simple ability to form a water-insoluble amine polymer salt does not in itself provide definitive identification of microcapsule wall-forming components, at least under the conditions tested to date.

To determine whether an amine/polymer pair will form capsules and microcapsules, the following procedure is useful. Prepare separate aqueous solutions of the amine and polymer, containing about 1% w/v of the acid form of the polymer and an approximately stoichiometrically equivalent amount of amine, in equal volumes of water. Alternatively, if the polymer or amine are not soluble to such an extent, prepare separate solutions of water-soluble salts of the amine (*e.g.*,

hydrochloride or acetate) and of the polymer (e.g., sodium or ammonium). To an approximately 5 ml volume of the amine solution add successive 20 to 25 microliter volumes of the polymer solution, delivering the polymer solution dropwise from a height of about 1 centimeter. Visually observe the two solutions as the one is added to the other. Note whether the droplets of polymer solution merge with the amine solution and the system becomes homogeneous or whether a pellicle forms about the polymer solution droplets and keeps them as physically distinct and mechanically separate entities.

If the added droplets form such a pellicle and do not blend with the amine solution to make a homogeneous solution, it is probable the reactant pair can be used to make microcapsules. To test this probability more closely, it is necessary to repeat the experiment using polymer droplets and amine solutions prepared over a range of concentrations to establish optimal reactant concentrations.

If either the amine or acid form of the polymer is inadequately soluble to conduct the test as described above, the salt forms of the reactant pair may be used together in their place.

FIGURE 3 indicates which amine polymer salts have effectively formed stable microcapsules, under conditions tested to date.

The ability of a combination of aqueous solutions of amine and polymer to form a stable microcapsular configuration requires first that the reactants be water-soluble and oppositely charged in order that they may combine to form a poorly soluble salt. Importantly, the anionic polymer strands in solution must not rapidly diffuse compared to the ability of the amine molecules (ions) to diffuse. Further, it is preferred that the droplets of anionic polymer solution be introduced into the amine solution in such a manner that the polymer droplets do not become extensively distorted or very rapidly mixed with the bulk of the amine solution. These several requirements are relatively easily met. The basis for these requirements may be understood in terms of the steps in the process described in greater detail below.

At the start of the microcapsule-making process, the aqueous solutions of anionic polymer and of the amine are mechanically (i.e., physically) separated phases. At room temperature, the water molecules of the anionic polymer solution are expected to have high (0.9+) thermodynamic activity coefficients and to diffuse far more rapidly than do the polymer molecules. The polymer strands (molecular weights above 10 kD, 100,000 AMU) are of colloidal size and can be expected to behave as do other colloidal particles. In particular, it is expected that a colloidal solution of anionic polymer will tend to structural inhomogeneity with development of microregions of relatively high colloidal polymer concentration and others void of polymer. This behavior of colloidal polymers has recently been shown by Ito *et al.*, 1994, *Science* 263:66-68, with time lapse confocal laser micrographs. The micrographs show such tendency toward inhomogeneity and void structure. Ito *et al.* discuss this behavior in terms of ionic polymers such as those employed in this invention (e.g., sodium polyacrylate). By contrast, amine reactants (molecular weights less than 400) are thermodynamically far more active than are the anionic polymer strands, diffuse far more

rapidly than do the polymer strands (but more slowly than do water molecules), and are understood to be homogeneously distributed throughout their solutions.

When a droplet of anionic polymer solution is introduced into a bulk volume of amine solution it is expected that the previously separate aqueous phases will essentially instantaneously combine to form a single continuous aqueous phase with no discernible phase boundary for the previously separate aqueous components. On the other hand, the low diffusion coefficients of the anionic polymers (typically less than 7×10^{-7} cm²/sec for colloidal polymers in the mass range near 10 kD) limit movement of polymer molecules from their initial positions relative to the remainder of the polymer solution droplet and allow time for a multiplicity of amine molecules (ions), moving nearly as rapidly as water molecules, to come into proximity with the polymers, to be electrostatically attracted to them, and to form salts with anionic groups of the polymers. Thus, the relative immobility of the polymer molecules during reaction with numerous amine units allows precipitation of a shell which conforms to the approximate initial positions of the polymer strands and retains the shape of the droplet.

The development of this shell around a droplet may be easily observed macroscopically by carefully adding an approximately 20 microliter, approximately spherical droplet of aqueous 1% w/v sodium carboxymethylcellulose solution to an approximately 1% w/v aqueous solution of decylamine hydrochloride. Within a small fraction of a second, a barely discernible spherical pellicle forms around the added droplet and, during a few more seconds, the pellicle becomes increasingly thicker and more opalescent. The resulting microcapsule can be retrieved with a Pasteur pipette or collected on fine netting. It should be noted that, if such a polymer droplet is delivered to the amine solution from a height of several centimeters, it is highly probable that the droplet may be distorted to form an oblate spheroid or biconcave disk-like structure which similarly gradually becomes thicker and more opalescent of shell. If the amine solution is stirred or rapidly flowing, added droplets tend to form prolate spheroids or filamentous particles which similarly thicken. If the droplets of polymer solution are smaller, they may be dispersed onto or into the amine solution from greater heights or onto/into flowing amine solution with less distortion. In practice, droplets of approximately 5 to 7 micron diameter may be applied from a height of 5 centimeters to the surface of an amine solution flowing at linear velocity of about 1 centimeter per second and still produce essentially spherical microcapsules.

While it is perhaps too simple to describe the process by which pellicles surrounding polymer droplets form and thicken to make microcapsules in terms only of diffusional processes, such a description conveys a fairly accurate sense of what happens. A more detailed understanding may be derived through the description of the mechanism and dynamics of ion transport across a liquid-liquid interface. Benjamin, I., 1993, *Science* 261:1558-1560, has shown that, although the time averaged water-dichloroethane interface is "molecularly sharp", over short time intervals, thermal fluctuations induce formation of capillary interdigitations of each liquid phase with the other. These capillary "fingers" allow ion transfer from one phase to another even though the bulk phases are clearly separate. It is to be expected that similar capillary intrusions of an

amine-bearing part of the aqueous phase into polymer-bearing aqueous phase might similarly provide a mechanism by which the ions of the amine polymer salt might interact to develop a film or pellicle without grossly disturbing the integrity of the polymer droplet.

In effect, then, the ability of the reactant solutions to form discrete microcapsules depends at least in part on the relative immobility of polymer strands in aqueous media and the relatively much greater mobility of amine molecules (ions), and perhaps also in part on the brief thermally induced fluctuation of the apparent interfacial boundary between amine and polymer solutions. It does not require high viscosity solutions, but rather one species of slow diffusing reactant. This interpretation of the mechanism of microcapsule formation is quite at odds with the constraints on microcapsule formation put forth in other entirely aqueous encapsulation systems.

The reaction leading to formation of amine polymer salt precipitates may be seen to be a simple salt exchange and as such has the characteristics of a reversible reaction. That this is so is demonstrable by adding an excess of the soluble salt formed in the reaction, or a concentrated solution of it (*e.g.*, sodium chloride or sodium acetate), to a suspension of microcapsules. Raising the concentration of sodium chloride in the aqueous medium surrounding a population of microcapsules to about 4% w/v generally results in their rapid dissolution. However, treatment with sodium chloride or another electrolyte capable of yielding soluble polymer and amine salts (*e.g.*, sodium phosphate to make a 4% w/v solution) may not completely disrupt microcapsules made with very poorly soluble amines (*e.g.*, hexadecylamine, octadecylamine), and, to disrupt such microcapsules, *e.g.*, for analytical purposes, it is useful to add a solvent that is able to deplete the aqueous concentration of amine (*e.g.*, cyclohexane).

A presently preferred method for forming the microcapsules of the invention is to employ an acoustical droplet-forming device that has been developed for this purpose. This device produces a stream of uniform fine droplets of anionic polymer solution and directs them onto and through a constantly renewed surface of the cationic reactant solution (amine) so that newly arriving droplets do not impinge on earlier delivered droplets. The device thereby (1) reduces the tendency to form microcapsule agglomerates and (2) provides a means to produce large populations of microcapsules with a very narrow size distribution range. The machine operates by sonically pulsing a downward flowing vertical stream of polymer solution just before it emerges from a narrow orifice so that the sound wave propagating through the liquid stream initiates a series of constrictions in the stream which then, under the influence of the surface tension of the liquid, causes the stream to break up into a train of uniform droplets. The droplet train is directed coaxially into a narrow cylindrical tube which is supplied through a side opening (or its topologic equivalent) with a continuous flow of the cationic reactant. Thus, each newly arriving polymer droplet encounters a fresh surface of cationic reactant and has minimal opportunity to strike and coalesce with another polymer droplet before it begins to form its own capsular wall and exits the lower end of the tube.

The major components of the acoustic device for preparation of microcapsules may perhaps be best described in terms of its functional sequence as it brings two liquid streams

together to form microcapsules. In this device, aqueous solutions of anionic polymer and amine (or their corresponding neutral salts) are stored in separate reservoirs and pumped through separate transfer lines. The amine solution is fed to and enters the stem of a modified T-tube which serves as the primary reaction vessel. The T-tube is mounted so that the cylindrical axis of the bar of the T is oriented vertically. Amine solution entering the stem of the T-tube flows horizontally for a few millimeters before it flows by gravity out the lower half of the T-tube bar. (In practice, it has been useful to employ not a simple T-tube but rather one of the sort often referred to in clinical chemical laboratories as a "cactus tube". A cactus tube has the general shape of the lower case letter h and, in this application, the tube is positioned so that the h shape is upside down. The straight part of the cactus tube is about 2 cm long and has an internal diameter of about 2 mm.) Amine solution flowing from the T-tube may be returned to the reservoir and recirculated.

The polymer solution is pumped through a membrane filter (of 8 micron or finer retentiveness), then through a glass capillary, the distal end of which is constricted to a nominal diameter of 20 to 25 microns, and emerges in the form of a fine continuous liquid jet. (The constricted capillary is readily fabricated from a volumetric 25, 50 or 100 microliter glass capillary tube of a type generally available from laboratory supply houses, e.g., A.H. Thomas Co.) The constriction is preferably such that the jet of polymer solution emerges with a velocity in the range between 4 and 5 meters per second when the polymer solution is pumped at 1 to 2 ml per minute, but flow rates and velocities outside these ranges may, of course, be employed.

The capillary is aligned in a shallow V-shaped groove in a metal block and tightly held by compression springs against the axially vibrating end of an acoustic transducer (e.g., of a laboratory ultrasonic probe operated at a nominal energy output of 40 watts) so that acoustic energy is transferred through the wall of the capillary to the flowing polymer solution, causing the jet of polymer solution to break up into a train of droplets of uniform size.

The transducer-capillary-compression block assembly is positioned so that the emerging train of polymer solution droplets passes through air for about 3 cm and is directed axially into the upper end of the T-tube bar to impinge on the amine solution entering from the side (stem) of the T-tube. The polymer solution droplets react with the amine solution to form microcapsules that flow with the amine solution out the lower end of the T-tube bar.

Even in the absence of sonic stimulation, the jet of polymer solution which emerges from the capillary constriction, as described above, would normally spontaneously break up into a train of droplets of varying size as a result of varying natural instabilities of the fluid stream and of the atmosphere into which the jet emerges, the so-called Rayleigh disruption of a liquid jet. However, it is desirable that droplets of uniform size be produced in order to make microcapsules of uniform size. It is for this reason that, in accordance with the preferred embodiment of this invention, droplets of uniform size are produced by periodically sonically disturbing the liquid stream to initiate a train of sufficiently strong compression (sound) waves along the axis of the jet. The train of sound waves moves through the liquid medium and away from the orifice far more rapidly than the liquid itself. (The jet emerges at a velocity of 4 to 5 meters per second.) Propagation of the wave

train along the length of the jet establishes an interference pattern of increasing constructive amplitude at successive nodes along the path of the jet. At some distance from the orifice, the amplitude of the surface wave becomes greater than the surface tension of the liquid, and droplets of polymer solution form at the sonicator frequency. The generation of a train of droplets of uniform size in this manner is reported in some detail by Galley, P.J., and Hieftje, G.M., 1992, *Applied Spectroscopy* 45:1460-1463.

To illustrate the foregoing method and apparatus, reference is made to the accompanying FIGURE 4, in which is shown "h" shaped tubular member **10**, including a side-leg entry segment **14** and a vertical intersecting segment **12**, through which the amine solution **16** is pumped downwardly at the upper end of tube segment **14** so that it enters upright segment **12** and is diverted at the intersection of segment **14** and segment **12** into a downwardly flowing portion from which it exits at the bottom end of tubular segment **12**. Above the intersection of segments of **14** and **12**, the polymer solution **22** is introduced through a capillary member **18**, the bottom end of which is spaced a predetermined distance (about 3 centimeters in the exemplary description above) above the intersection of segments of **14** and **12**, at which the flow of the amine solution is diverted downwardly, so that drop-wise disposed portions of polymer solution **22** are combined with the downwardly-flowing amine solution at that point.

As described above, to enhance the uniformity of the drop-wise, downwardly-flowing portions **22** of polymer solution, capillary tube **18** is rigidly held in a metal block **24**, with a V-groove holding slot (not shown in the figure) and acoustically stimulated intermittently. For that purpose, an acoustic probe **20** is in contact with capillary tube **18** near the lower end thereof.

One may estimate the numbers of individual droplets produced per unit time from the frequency of the sonicator. In most instances, a sonicator with a frequency of 20 kHz was employed. The estimate of the number of droplets formed may be slightly in error due to instances in which successive droplets may impinge on one another and coalesce or adhere to one another to form aggregated microcapsules. In practice, far less than 1% of the droplets occur as fused or coalesced forms. Assuming all droplets are formed separately, one may calculate the size of individual droplets from a knowledge of polymer flow rate. At a nominal anionic polymer flow rate of 1 ml per minute, the volume of individual droplets is 0.05 microliters (cubic millimeters), corresponding to a spherical droplet diameter of 4.57 microns. The diameter of microcapsules formed at flow rates near 1 ml per minute and acoustic frequencies of 20 kHz is approximately 5 microns as estimated from volume diameter sizing (Coulter principle).

Alternatively, droplets of the polymer solution of essentially any size may be introduced into the cationic reactant solution (e.g., by spraying, or by dropwise dispensing from a pipette) to form microcapsules. In many applications it is desirable that the microcapsules formed be of highly uniform size, and thus some means of inducing this uniformity, such as the acoustic method described above, is preferred. Such applications include delivery to the lymphatic tissues of the intestine, often referred to as Peyer's patches. The M cells of Peyer's patches preferentially reject

particles larger than about 10 microns, but engulf particles in the size range below about 10 microns and transfer them to other lymphatic cells.

In general, the microcapsules of the present invention may range in size from 0.1 to 2,000 microns. A preferred size range, useful for general oral administration, is from 500 to 1,000 microns. In some embodiments, the range is from 100 to 200 microns. In other embodiments, such as in the administration of substances intended for delivery to Peyer's patches in the lymphatic tissue of the intestine, a particularly preferred size range is from 1 to 10 microns.

Depending in part on the degree to which manufacturing fluid is removed and in part on the nature of the core solute, the aqueous core microcapsules may be collected as a free-flowing suspension, a viscid flowable concentrate, a paste, a friable flake or, with further treatment, as a lyocake. Lyophilization is particularly desired to provide stable microcapsules with highly water-soluble core materials.

Once encapsulated, immunogenic compositions are protected from the environment, but may be slowly released from the microcapsules by suspending the capsules in an aqueous medium into which the immunogenic compositions can actively diffuse through the semi-permeable microcapsule walls. In general, where the nature of the wall-forming reactants is held constant, highly water-soluble immunogenic compositions are observed to be released more rapidly than are poorly water-soluble immunogenic compositions and, in general, immunogenic compositions of low molecular weight are released more rapidly than are those of higher molecular weight. In some embodiments, conversion of the microcapsules to lyocakes and resuspension in aqueous media is preferred.

Vaccines:

Vaccines according to the invention comprise at least one microencapsulated immunogenic composition and a pharmaceutically or veterinarily acceptable carrier or diluent. Optionally, the vaccine may comprise additional components, including microencapsulated and non-encapsulated immunogenic compositions and/or adjuvants.

Thus, the present invention provides a vaccine for protecting a member of a bovine species against BHV-1, comprising an immunogenic amount of microcapsules comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and an immunogenic composition surrounded by the salt membrane, said microcapsules being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of a selected water-soluble anionic polymer with a selected water-soluble amine, or the interfacial reaction of a water-soluble neutral salt of a selected anionic polymer with a water-soluble neutral salt of a selected amine. In a preferred embodiment, the selected anionic polymer is alginic acid, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., sodium alginate), and the selected amine is spermine, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., spermine hydrochloride).

-21-

The immunogenic composition comprises a subunit component of BHV-1 which is capable of inducing a protective response against BHV-1 when administered in encapsulated form according to the present invention to a member of a bovine species. In a preferred embodiment, the subunit component of the immunogenic composition of the vaccine comprises a BHV-1 glycoprotein such as, e.g., gI, gIII or gIV, or an immunogenic fragment or derivative thereof. In a more preferred embodiment, the immunogenic composition of the vaccine
10 comprises a truncated BHV-1 glycoprotein, such as e.g., BHV-1-gD. In a more preferred embodiment, the immunogenic composition of the vaccine is BHV-1-gD. The vaccine may further comprise a pharmaceutically or veterinarily acceptable carrier or diluent, and, optionally, an adjuvant. As used herein, the term "member of a bovine species" refers to cows and oxen.

The phrase "capable of inducing a protective response" is used broadly herein to include the induction of any immune-based response in the animal in response to
20 vaccination, including either an antibody or cell-mediated immune response, or both, that protects the vaccinate against BHV-1. The term "protective response" as used herein is not limited to absolute prevention of infection by BHV-1, but is intended to include any reduction in infectivity of the virus, or in the severity of a disease or condition normally resulting from infection with BHV-1, including a detectable reduction in one or more of the pathological effects or

-21a-

symptoms normally resulting from infection, or a detectable reduction in the rate of progression of one or more of such pathological effects or symptoms, compared to an unvaccinated, infected animal.

Vaccines of the present invention may be formulated following accepted convention using standard buffers, carriers, stabilizers, diluents, preservatives, and solubilizers, and may also be formulated to facilitate sustained release. Diluents may include water, saline, dextrose, ethanol, glycerol, and the like. Additives for isotonicity may include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin, among others. Adjuvants may be employed, examples of which include the RIBI adjuvant system (Ribi Inc.), alum, aluminum hydroxide gel, oil-in-water emulsions, water-in-oil emulsions such as, e.g., Freund's complete adjuvant and Freund's incomplete adjuvant, Block copolymer (CytRx, Atlanta GA), QS-21 (Cambridge Biotech Inc., Cambridge MA), SAF-M (Chiron, Emeryville CA), AMPHIGEN[®] adjuvant, Quil A, monophosphoryl lipid A, and Avridine lipid-amine adjuvant. The vaccine may further comprise one or more other immunomodulatory agents such as, e.g., interleukin-1 or other known cytokines. Suitable other vaccine vehicles, carriers and additives are known, or will be apparent to those skilled in the art. See, e.g., Remington's Pharmaceutical Science, 18th Ed., 1990, Mack Publishing. Vaccines may be maintained in solution or lyophilized. Lyophilized vaccine may be stored

-21b-

conveniently and reconstituted with a sterile diluent solution prior to administration.

The amount of microencapsulated immunogenic composition administered to the animal depends upon such factors as the nature of the immunogenic composition, the species, age, weight, and general physical characteristics of the animal being vaccinated, and the composition of the vaccine. Determination of optimum dosage for each parameter may be made by routine

methods in view, e.g., of published studies. Specifically regarding administration of microencapsulated subunit components of BHV-1, and, in particular, microencapsulated BHV-1-gD, the amount administered to a member of a bovine species will preferably range from about 100 pg to about 150 μ g of BHV-1-gD, more preferably from about 250 pg to about 100 μ g, and most preferably from about 400 pg to about 60 μ g. The typical dose volume of the vaccine will range from about 0.5 ml to about 5 ml per dose per animal.

The vaccine regimen may also be selected based on the above-described factors. Animals may be vaccinated at any time, including at weaning age or younger, or just prior to or at the time of breeding, or at the time at which onset of infection in a herd is first detected. Supplemental administrations, or boosters, may be required for full protection. One method of determining whether adequate immune protection has been achieved is to determine seroconversion. This and other methods for determining whether adequate immune protection has been achieved are well-known in the art.

The present invention also provides a method of vaccinating a member of a bovine species against BHV-1, comprising administering a vaccine comprising an immunogenic amount of the microcapsules of the present invention. Vaccines according to the invention may be administered by any appropriate route such as, e.g., by oral, intranasal, intramuscular, intraperitoneal, intravenous, intra-arterial, subcutaneous, rectal or vaginal administration, or by a combination of routes.

In addition, the present invention provides a kit for vaccinating a member of a bovine species against BHV-1, comprising a first container comprising an immunogenic amount of the microcapsules of the present invention. The kit further comprises a second container comprising a pharmaceutically or veterinarily acceptable carrier or diluent. The microcapsules of the kit may optionally be supplied in lyophilized form. The kit may also optionally comprise an adjuvant.

The present invention further provides a combination vaccine for protecting a member of a bovine species against BHV-1 and, optionally, another disease or pathological condition which afflicts a bovine species, which vaccine comprises an immunogenic amount of microcapsules comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and a first immunogenic composition surrounded by the salt membrane, said microcapsules being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of a selected water-soluble anionic polymer with a selected water-soluble amine, or the interfacial reaction of a water-soluble neutral salt of a selected anionic polymer with a water-soluble neutral salt of a selected amine. In a preferred embodiment, the selected anionic polymer is alginic acid, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., sodium alginate), and the selected amine is spermine, which is preferably in the form of a corresponding water-soluble, neutral salt (e.g., spermine hydrochloride).

The first immunogenic composition comprises a subunit component of BHV-1 capable of inducing a protective response against BHV-1 when administered in encapsulated form according

-23-

to the present invention to a member of a bovine species. In a preferred embodiment, the first immunogenic composition comprises a BHV-1 glycoprotein such as, e.g., gI, gIII or gIV, or an immunogenic fragment or derivative thereof. In a more preferred embodiment, the first immunogenic composition comprises a truncated BHV-1 glycoprotein, such as e.g., BHV-1-gD. In a more preferred embodiment, the immunogenic composition is BHV-1-gD.

10 The combination vaccine further comprises an immunogenic amount of a second immunogenic composition, which is selected based on its ability to induce a protective response against either BHV-1 or another disease or pathological condition which afflicts a bovine species, as known in the art. Any immunogenic composition known to be useful in a bovine vaccine composition may be used as the second immunogenic composition of the combination vaccine of the present invention. Such immunogenic compositions include but are not limited to those that provide protection against
20 bovine respiratory syncytial virus, bovine viral diarrhea virus, parainfluenza virus types I, II, or III, *Leptospira* spp., *Vibrio fetus*, rotavirus, coronavirus, rabies, *Pasteurella hemolytica*, *Pasteurella multocida*, *Clostridia* spp., Tetanus toxoid, *E. coli*, and *Neospora* spp., among others. The second immunogenic composition of the combination vaccine may either be encapsulated by the method described herein, or by any other known method, or may remain non-encapsulated. If encapsulated, the second immunogenic

-24-

composition may either be encapsulated in the same microcapsules with the BHV-1 subunit component, or may be encapsulated separately in a different set of microcapsules.

The immunogenic component of the second immunogenic composition may optionally be linked, covalently or otherwise, to the BHV-1 subunit component, or to an immunogenic fragment or derivative thereof, to produce a chimeric molecule. In one embodiment, the component of the second immunogenic composition comprises a hapten, the immunogenicity of which is
10 detectably increased by conjugation to the BHV-1 subunit component of the first immunogenic composition.

Chimeric molecules comprising components of the first and second immunogenic compositions described above may be synthesized using one or more techniques known in the art. For example, a chimeric molecule may be produced synthetically using a commercially available peptide synthesizer utilizing standard chemical synthetic processes (see, e.g., Merrifield, 1985, Science 232:341-347). Alternatively, a chimeric
20 molecule may be produced using recombinant DNA technology, as known in the art, whereby, e.g., nucleotide sequences encoding the different components of the chimeric molecule are spliced together in-frame and expressed in a suitable transformed host cell for subsequent isolation. Ample guidance for carrying out such recombinant techniques is provided in Maniatis, et al., 1989, supra; Ausubel, et al., 1989, supra; Sambrook, et al., 1989, supra; and Innis et al. (eds) 1995, supra.

The combination vaccine may further comprise a

-24a-

pharmaceutically or veterinarily acceptable carrier or diluent, and optionally, an adjuvant.

The following example is illustrative only, and does not limit the scope of the present invention.

EXAMPLE

The capacity of microencapsulation to enhance virus-specific immune responses to BHV-1-gD was determined. Also examined was the importance of both protein capture efficiencies and stability of captured proteins within
10 microcapsules in enhancement of virus-specific immune responses.

Materials and Methods

Virus and viral proteins:

BHV-1-gD at a concentration of 696 µg per ml was provided by Pfizer Inc. Plasmid constructions, plasmid transfections, and expression, purification, and quantitation of BHV-1-gD were performed as described by Drunen, S., et al., 1994, Vaccine 12:1295-1302.

Formation of Microcapsules:

20 Microencapsulation of BHV-1-gD using spermine-alginate or spermine-chondroitin sulfate was performed as follows. Separate aqueous 1% w/v solutions of high viscosity sodium alginate (Fisher Scientific Co., Fairlawn, NJ, or Kelco, Clark, NJ), or sodium chondroitin sulfate C (Sigma Chemical Co., St. Louis, MO) were allowed to hydrate at refrigerator temperature overnight, and after hydration were passed through 8 micron mixed cellulose ester filters (Millipore, Bedford,

-24b-

MA). Volumes of 2.5 ml of vaccine and successive half volumes to 0.078 ml were transferred with the aid of a calibrated pipette to 0.6 ml volumes of 1% w/v sodium alginate solution and to 5 ml volumes of sodium chondroitin sulfate solution in separate vessels. Each vaccine-polymer mixture was diluted to 10 ml, mixed by vortexing and, using the previously taught acoustical droplet-forming device, instilled as a stream of uniform fine droplets into separate 40 ml volumes of aqueous 0.125% w/v spermine (Sigma Chemical Co., St. Louis, MO) adjusted to pH 7.0 ± 0.1 with hydrochloric acid.

In order to minimize the opportunity for polymer droplets entering the spermine hydrochloride solution in the cactus tube to impact upon nascent capsules, the spermine hydrochloride solution was delivered to the cactus tube at a rate of about 9 ml per minute and the sodium chondroitin sulfate solution was pumped to the capillary orifice at a rate of about 1 ml per minute. Spermine alginate microcapsules undergo substantial syneresis. In order to obtain microcapsules in the median size range of 5 microns, the sodium alginate solution was delivered to the capillary orifice at a nominal rate of 2 ml per minute.

Microencapsulation using decylamine-carboxymethyl-cellulose (CMC) was performed as follows. Sodium carboxymethylcellulose N.F., medium viscosity, (Ruger Chemical Co., Fairlawn, N.J) and solutions of it were allowed to hydrate for at least 24 hours before use. Decylamine (Aldrich Chemical Co., Milwaukee, WI) was solubilized in water by

-24c-

titration to pH 7.0 with acetic acid (Fisher Scientific Co., Pittsburgh, PA). Decylamine-CMC microcapsules containing BHV-1-gD were similarly generated by thoroughly mixing graded volumes of vaccine with separate 0.75 ml

volumes of 1% w/v sodium carboxymethylcellulose and enough water to make 5 ml, and acoustically pulsing the resulting mixture into 20 ml of 0.5% w/v decylamine adjusted to pH 7.0 ± 0.1 with acetic acid.

Sodium alginate and decylamine carboxymethylcellulose microcapsule formulations were washed three times; those of spermine chondroitin sulfate once by centrifugation (20,000 gxm), decanting, resuspending in water, and again centrifuging.

To determine capture efficiencies for BHV-1-gD, microcapsules composed of either spermine-alginate or decylamine-CMC were disrupted with 8 M sodium phosphate buffer, and those composed of spermine-chondroitin were disrupted with 1% sodium chloride. Quantities of captured antigen were compared with quantities of starting material to determine capture efficiencies.

To determine how long viral proteins were contained within microcapsules, BHV-1-gD was microencapsulated in either spermine-alginate or spermine chondroitin sulfate and stored at 4°C for 42 days. Aliquots of microcapsules were taken at various intervals after formation, washed, and tested for the presence of captured antigen by disrupting samples as described above.

ELISA to detect BHV-1-gD:

Individual wells of flat-bottomed, 96-well plates (MAXISORP[®], Nunc, Naperville, IL.) were coated with 100 μl of rabbit anti-BHV-1-gD diluted 1:10,000 in a sodium borate solution and incubated overnight in a humidified chamber at 4°C . Wells were washed 4 times with 300 μl PBS containing 0.5% Tween 20 (Sigma, St. Louis, MO.) (PBST). Four serial 2-fold dilutions of samples to be tested (diluted in PBST) were added in a volume of 100 μl per well and incubated for 2 hours at 37°C . Experimental samples were compared with a standard quantity of BHV-1-gD. Eight serial 2-fold dilutions from 1:1,600 to 1:204,800 of the BHV-1-gD standard were performed. Wells containing 1 μg of rotavirus (bovine strain WC3) were used as negative controls. Wells were washed 4 times with PBST, and 100 μl of mouse anti-BHV-1-gD ascitic fluid diluted 1:4,000 in PBST were added to each well and incubated for 90 min at 37°C . Wells were washed 4 times with PBST, and 100 μl of HRP-labeled sheep anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN.) diluted 1:2,000 in PBST plus 1% normal rabbit serum (Sigma) were added to each well and incubated for 90 min at 37°C . Wells were washed 4 times with PBST, and developed with 100 μl per well of peroxidase substrate (ABTS, Kirkegaard & Perry Laboratories, Gaithersburg, MD.). Colorimetric changes were determined at an optical density (OD) of 405 nm on a microplate ELISA reader (Dynatech MR4000, Dynatech Labs., Chantilly, VA.). The reaction was considered complete when the BHV-1-gD standard diluted 1:12,800 was 1.0 (+/- 0.1) OD units.

To determine geometric mean titers and standard errors, samples with undetectable BHV-1-gD-specific antibody responses were assigned a value of 10.

Inoculation of mice:

Groups of 5 adult, Swiss-Webster female mice (Taconic Breeding Laboratories, Germantown, N.Y.) were inoculated with various doses of either microencapsulated or unencapsulated preparations of BHV-1-gD intramuscularly (by quadriceps femoris) in a volume of 50 µl per leg. Blood was obtained by retro-orbital capillary plexus puncture using non-heparinized Natelson tubes (Monoject Corp., St. Louis, MO.) at 1 and 2 months after inoculation, and tested for the presence of BHV-1-gD-specific antibodies by ELISA, as described below.

ELISA assay to detect BHV-1-gD-specific IgG:

Individual wells of flat-bottomed, 96-well plates (IMMULON® 2, Dynatech Labs, Chantilly, VA.) were coated with 50 µl containing 100 ng per well of BHV-1-gD diluted in PBS and stored in a humidified chamber at 4°C overnight. Plates were washed once with dH₂O, and 300 µl of 1% poly(vinyl alcohol) (PVA) (Aldrich Chemical Co., Milwaukee, WI.) were added to each well and incubated for 2 hours at 37°C. Plates were washed 5 times with dH₂O, and 100 µl per well of serum samples (diluted 1:50 in 1% PVA) were added to each well and plates were incubated for 1 hour at 37°C. Serum from a mouse hyperimmunized with BHV-1-gD was diluted serially 2-fold from 1:200 to 1:204,800, and used as a positive control. Plates were washed 5 times with dH₂O, and 100 µl per well of HRP-labelled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD.) diluted 1:10,000 in 1% PVA were added and incubated for 1 hour at 37°C. Plates were washed 5 times with dH₂O, and developed with 100 µl per well of peroxidase substrate (ABTS, Kirkegaard & Perry Labs.). Colorimetric changes were determined at an OD of 405 nm on a microplate ELISA reader after a 15 min incubation. BHV-1-gD-specific antibody titers were calculated by comparison with a standard curve generated using hyperimmune antisera of known titer.

Results

The quantity of BHV-1-gD required to induce BHV-1-gD-specific antibody responses was reduced approximately 5,000-fold by microencapsulation in spermine-alginate and 3.3-fold by microencapsulation in decylamine-CMC (TABLE 1). BHV-1-gD-specific immune responses were not enhanced after microencapsulation in spermine-chondroitin sulfate.

Capture efficiencies were not related to enhancement of antigen-specific immune responses. Microcapsules composed of decylamine-CMC were about 20 times more efficient at capturing BHV-1-gD, but 1,500 times less capable of enhancing BHV-1-gD-specific antibody responses, as compared with spermine-alginate microcapsules containing BHV-1-gD (TABLES 1 and 2). In addition, although there were no differences in the efficiencies of capture among preparations of BHV-1-gD in spermine-alginate or spermine-chondroitin sulfate, only spermine-alginate microcapsules containing BHV-1-gD enhanced antigen-specific immune responses.

-27-

BHV-1-gD was captured and retained within both spermine-alginate and spermine-chondroitin sulfate for at least 42 days (TABLE 3). Therefore, differences in enhancement of antigen-specific immune responses could not be accounted for by rapid leakage of viral proteins from microcapsules as observed in vitro.

10 It has now been demonstrated that a BHV-1 subunit component, in this case BHV-1-gD, can be successfully captured within the matrix of microcapsules of the invention, and that microencapsulation of the BHV-1 subunit component greatly enhances its immunogenicity.

The present invention is not to be limited in scope by the specific embodiments described, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent compositions and methods are within the scope of the invention.

TABLE 1. Humoral immune responses to encapsulated or unencapsulated preparations of BHV-1-gD in mice.

5	Immunogen	Dose ^a	Microcapsular material	GMT ^b (+/- SE) of BHV-1-gD-specific antibody post-inoculation	
				1 month	2 months
10	BHV-1-gD	2 µg	none	30 (+/-400)	10 ^c
		400 ng		10	10
		80 ng		10	10
15	BHV-1-gD	10 ng	Spermine-alginate	8,800 (+/-900)	10,200 (+/-2600)
		2 ng		5,800 (+/-1600)	5,800 (+/-1600)
		400 pg		300 (+/-500)	200 (+/-600)
20	BHV-1-gD	10 ng	Spermine-chondroitin sulfate	10	10
		2 ng		10	10
		400 pg		10	10
25	BHV-1-gD	600 ng	Decylamine-CMC	2,200 (+/-500)	30 (+/-400)
		60 ng		10	10
		6 ng		10	10

^a Data collected from groups of 5 mice for each dose for each treatment.

^b GMT = geometric mean titer

^c To determine GMT and SE, samples with undetectable BHV-1-gD-specific antibody responses were assigned a value of 10.

TABLE 2. Relative capacities of different microcapsular materials to capture BHV-1-gD.

Antigen	Microcapsular material	Amt. antigen available	Amt. antigen captured	Capture efficiency
BHV-1-gD	Spermine-alginate	46 µg	256 ng	0.56%
		31.5 µg	156 ng	0.5%
		18 µg	126 ng	0.7%
		9.4 µg	34 µg	0.36%
BHV-1-gD	Spermine-chondroitin sulfate	63 µg	103 ng	0.16%
		31 µg	115 ng	0.37%
		15 µg	59 ng	0.39%
		7.6 µg	41 ng	0.54%
BHV-1-gD	Decylamine-CMC	11 µg	1.4 µg	12.7%

5 TABLE 3. Long-term stability of BHV-1-gD in microcapsules.^a

Antigen	Microcapsular material	Amt. antigen remaining in microcapsules at various intervals (days) after formation						
		0	1	3	7	14	28	42
BHV-1-gD	Spermine-alginate	12 ng	12 ng	12 ng	10 ng	8 ng	6 ng	4 ng
BHV-1-gD	Spermine-chondroitin sulfate	38 ng	36 ng	38 ng	38 ng	36 ng	35 ng	34 ng

^a After capture in microcapsules, amount of antigen remaining at various intervals was determined by disruption of aliquots of microcapsules.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: PFIZER INC., THE CHILDREN'S HOSPITAL OF PHILADELPHIA and TEMPLE UNIVERSITY

10 (ii) TITLE OF INVENTION: AQUEOUS SOLVENT BASED ENCAPSULATION OF A BOVINE HERPES VIRUS TYPE-1 SUBUNIT VACCINE

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

20 (A) ADDRESSEE: Pfizer Inc
(B) STREET: 235 East 42nd Street
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: United States of America
(F) ZIP: 10017-5755

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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30 (A) APPLICATION NUMBER: 2,229,430
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(C) CLASSIFICATION:

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(viii) ATTORNEY/AGENT INFORMATION:

40 (A) NAME: Smart & Biggar
(B) ADDRESS: P.O. Box 2999, Station D,
Ottawa, Ontario, Canada K1P 5Y6
(C) REFERENCE/DOCKET NUMBER: 65920-24

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (613)-232-2486
(B) TELEFAX: (613)-232-8440

(2) INFORMATION FOR SEQ ID NO:1:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1405 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

32

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 85..1335

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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    Val Thr Val Tyr Val Asp Pro Pro Ala Tyr Pro Met Pro Arg Tyr Asn
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    Ala Cys Asp Met Leu Ala Leu Ile Ala Asp Pro Gln Val Gly Arg Thr
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40  CTG TGG GAA GCG GTA CGC CGG CAC GCG CGC GCG TAC AAC GCC ACG GTC      399
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    ATA TGG TAC AAG ATC GAG AGC GGG TGC GCC CGG CCG CTG TAC TAC ATG      447
    Ile Trp Tyr Lys Ile Glu Ser Gly Cys Ala Arg Pro Leu Tyr Tyr Met
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    Glu Tyr Thr Glu Cys Glu Pro Arg Lys His Phe Gly Tyr Cys Arg Tyr
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60  GAG GGC CAG TAC CGA CGC GCG CTG TAC ATC GAC GGC ACG GTC GCC TAT      639
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33

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	His	Gly	Gly	Val	Val	Pro	Pro	Tyr	Phe	Glu	Glu	Ser	Lys	Gly	Tyr	Glu	
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20	CCG	CCG	CCT	GCC	GCC	GAT	GGG	GGT	TCC	CCC	GCG	CCA	CCC	GGC	GAC	GAC	927
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	AGC	CCC	GAC	GCC	GAC	CGC	CCC	GAA	GGC	TGG	CCG	AGC	CTC	GAA	GCC	ATC	1119
	Ser	Pro	Asp	Ala	Asp	Arg	Pro	Glu	Gly	Trp	Pro	Ser	Leu	Glu	Ala	Ile	
	330					335					340					345	
40	ACG	CAC	CCC	CCG	CCC	GCC	CCC	GCT	ACG	CCC	GCG	GCC	CCC	GAC	GCC	GTG	1167
	Thr	His	Pro	Pro	Pro	Ala	Pro	Ala	Thr	Pro	Ala	Ala	Pro	Asp	Ala	Val	
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	CCG	GTC	AGC	GTC	GGG	ATC	GGC	ATT	GCG	GCT	GCG	GCG	ATC	GCG	TGC	GTG	1215
	Pro	Val	Ser	Val	Gly	Ile	Gly	Ile	Ala	Ala	Ala	Ala	Ile	Ala	Cys	Val	
				365				370						375			
50	GCC	GCC	GCC	GCC	GCC	GGC	GCG	TAC	TTC	GTC	TAT	ACG	CGC	CGG	CGC	GGT	1263
	Ala	Ala	Ala	Ala	Ala	Gly	Ala	Tyr	Phe	Val	Tyr	Thr	Arg	Arg	Arg	Gly	
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	GCG	GGT	CCG	CTG	CCC	AGA	AAG	CCA	AAA	AAG	CTG	CCG	GCC	TTT	GGC	AAC	1311
	Ala	Gly	Pro	Leu	Pro	Arg	Lys	Pro	Lys	Lys	Leu	Pro	Ala	Phe	Gly	Asn	
		395					400					405					
	GTC	AAC	TAC	AGC	GCG	CTG	CCC	GGG	TGAGCGGCCT	AGGCCCTCCC	CCGACCGCCC	1365					
	Val	Asn	Tyr	Ser	Ala	Leu	Pro	Gly									
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60	CCTTTGCTCC	TAGCCCCGGC	TCCTGCCGAG	CCGCGCGGGG	1405												

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 417 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 20          25          30
Pro Ala Tyr Pro Met Pro Arg Tyr Asn Tyr Thr Glu Arg Trp His Thr
 35          40          45
20 Thr Gly Pro Ile Pro Ser Pro Phe Ala Asp Gly Arg Glu Gln Pro Val
 50          55          60
Glu Val Arg Tyr Ala Thr Ser Ala Ala Ala Cys Asp Met Leu Ala Leu
 65          70          75
Ile Ala Asp Pro Gln Val Gly Arg Thr Leu Trp Glu Ala Val Arg Arg
 85          90          95
30 His Ala Arg Ala Tyr Asn Ala Thr Val Ile Trp Tyr Lys Ile Glu Ser
 100         105         110
Gly Cys Ala Arg Pro Leu Tyr Tyr Met Glu Tyr Thr Glu Cys Glu Pro
 115         120         125
Arg Lys His Phe Gly Tyr Cys Arg Tyr Arg Thr Pro Pro Phe Trp Asp
 130         135         140
40 Ser Phe Leu Ala Gly Phe Ala Tyr Pro Thr Asp Asp Glu Leu Gly Leu
 145         150         155
Ile Met Ala Ala Pro Ala Arg Leu Val Glu Gly Gln Tyr Arg Arg Ala
 165         170         175
Leu Tyr Ile Asp Gly Thr Val Ala Tyr Thr Asp Phe Met Val Ser Leu
 180         185         190
Pro Ala Gly Asp Cys Trp Phe Ser Lys Leu Gly Ala Ala Arg Gly Tyr
 195         200         205
50 Thr Phe Gly Ala Cys Phe Pro Ala Arg Asp Tyr Glu Gln Lys Lys Val
 210         215         220
Leu Arg Leu Thr Tyr Leu Thr Gln Tyr Tyr Pro Gln Glu Ala His Lys
 225         230         235
Ala Ile Val Asp Tyr Trp Phe Met Arg His Gly Gly Val Val Pro Pro
 245         250         255
60 Tyr Phe Glu Glu Ser Lys Gly Tyr Glu Pro Pro Pro Ala Ala Asp Gly
 260         265         270

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35

Gly Ser Pro Ala Pro Pro Gly Asp Asp Glu Ala Arg Glu Asp Glu Gly
 275 280 285
 Glu Thr Glu Asp Gly Ala Ala Gly Arg Glu Gly Asn Gly Gly Pro Pro
 290 295 300
 Gly Pro Glu Gly Asp Gly Glu Ser Gln Thr Pro Glu Ala Asn Gly Gly
 305 310 315 320
 10 Ala Glu Gly Glu Pro Lys Pro Gly Pro Ser Pro Asp Ala Asp Arg Pro
 325 330 335
 Glu Gly Trp Pro Ser Leu Glu Ala Ile Thr His Pro Pro Pro Ala Pro
 340 345 350
 Ala Thr Pro Ala Ala Pro Asp Ala Val Pro Val Ser Val Gly Ile Gly
 355 360 365
 20 Ile Ala Ala Ala Ala Ile Ala Cys Val Ala Ala Ala Ala Ala Gly Ala
 370 375 380
 Tyr Phe Val Tyr Thr Arg Arg Arg Gly Ala Gly Pro Leu Pro Arg Lys
 385 390 395 400
 Pro Lys Lys Leu Pro Ala Phe Gly Asn Val Asn Tyr Ser Ala Leu Pro
 405 410 415
 30 Gly

-36-

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A microcapsule comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and an immunogenic composition surrounded by the salt membrane, said microcapsule being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of spermine and alginic acid or the interfacial reaction of a water-soluble neutral salt of spermine and a water-soluble neutral salt of alginic acid, said immunogenic composition comprising a subunit component of bovine herpes virus-1 (BHV-1) capable of inducing a protective response against BHV-1 when administered in microencapsulated form to a member of a bovine species.
2. The microcapsule of claim 1, wherein the water-soluble neutral salt of spermine is selected from the group consisting of halide and low molecular weight alkanolic salts, and the water-soluble neutral salt of alginic acid is selected from the group consisting of alkali metal salts, ammonium salts, and trialkanolamine salts.
3. The microcapsule of claim 2, wherein the water-soluble neutral salt of spermine is spermine hydrochloride and the water-soluble neutral salt of alginic acid is sodium

alginate.

4. The microcapsule of claim 1, wherein the subunit component of BHV-1 comprises a BHV-1 glycoprotein, or an immunogenic fragment or derivative thereof.

5. The microcapsule of claim 4, wherein the BHV-1 glycoprotein is selected from the group consisting of gI, gII, gIII and gIV.

6. The microcapsule of claim 4, wherein the subunit component comprises BHV-1-gD.

7. The microcapsule of claim 6, wherein the subunit component consists of BHV-1-gD.

8. A method of increasing the immunogenicity of a subunit component of BHV-1, comprising encapsulating said BHV-1 subunit component in a microcapsule comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core, said microcapsule being substantially free of non-aqueous contaminants, and salt membrane comprising a precipitate resulting from the interfacial reaction of spermine and alginic acid or the interfacial reaction of a water-soluble neutral salt of spermine and a water-soluble neutral salt of alginic acid.

-38-

9. The method of claim 8, wherein the water-soluble neutral salt of spermine is selected from the group consisting of halide and low molecular weight alkanolic salts, and the water-soluble neutral salt of alginic acid is selected from the group consisting of alkali metal salts, ammonium salts, and trialkanolamine salts.

10. The method of claim 9, wherein the water-soluble neutral salt of spermine is spermine hydrochloride and the water-soluble neutral salt of alginic acid is sodium alginate.

11. The method of claim 8, wherein the subunit component of BHV-1 comprises a BHV-1 glycoprotein, or an immunogenic fragment or derivative thereof.

12. The method of claim 11, wherein the BHV-1 glycoprotein is selected from the group consisting of gI, gII, gIII and gIV.

13. The method of claim 11, wherein the subunit component comprises BHV-1-gD.

14. The method of claim 13, wherein the subunit component consists of BHV-1-gD.

15. A vaccine for immunizing a member of a bovine species against BHV-1, comprising an immunogenic amount of

-39-

microcapsules comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and an immunogenic composition surrounded by the salt membrane, said microcapsules being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of spermine and alginic acid or the interfacial reaction of a water-soluble neutral salt of spermine and a water-soluble neutral salt of alginic acid, said immunogenic composition comprising a subunit component of BHV-1 capable of inducing a protective response against BHV-1 when administered in microencapsulated form to a member of a bovine species; and a veterinarily acceptable carrier.

16. The vaccine of claim 15, wherein the water-soluble neutral salt of spermine is selected from the group consisting of halide and low molecular weight alkanolic salts, and the water-soluble neutral salt of alginic acid is selected from the group consisting of alkali metal salts, ammonium salts, and trialkanolamine salts.

17. The vaccine of claim 16, wherein the water-soluble neutral salt of spermine is spermine hydrochloride and the water-soluble neutral salt of alginic acid is sodium alginate.

18. The vaccine of claim 15, wherein the subunit component of BHV-1 comprises a BHV-1 glycoprotein, or an

-40-

immunogenic fragment or derivative thereof.

19. The vaccine of claim 18, wherein the BHV-1 glycoprotein is selected from the group consisting of gI, gII, gIII and gIV.

20. The vaccine of claim 18, wherein the subunit component comprises BHV-1-gD.

21. The vaccine of claim 20, wherein the subunit component consists of BHV-1-gD.

22. The vaccine of claim 15, further comprising an adjuvant.

23. The vaccine of claim 22, wherein the adjuvant is selected from the group consisting of the RIBI and adjuvant system, alum, aluminum hydroxide gel, an oil-in-water emulsion, a water-in-oil emulsion, Block copolymer, QS-21, SAF-M, AMPHIGEN[®] adjuvant, Quil A, monophosphoryl lipid A, and Avridine lipid-amine adjuvant.

24. Use of a vaccine comprising an immunogenic amount of microcapsules comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and an immunogenic composition surrounded by the salt membrane, said microcapsules being substantially free of non-aqueous

-41-

contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of spermine and alginic acid or the interfacial reaction of a water-soluble neutral salt of spermine and a water-soluble neutral salt of alginic acid, said immunogenic composition comprising a subunit component of BHV-1 capable of inducing a protective response against BHV-1 when administered in microencapsulated form to a member of a bovine species, and a veterinarily acceptable carrier, in vaccinating a member of a bovine species against BHV-1.

25. The use of claim 24, wherein the water-soluble neutral salt or spermine is selected from the group consisting of halide and low molecular weight alkanolic salts, and the water-soluble neutral salt of alginic acid is selected from the group consisting of alkali metal salts, ammonium salts, and trialkanolamine salts.

26. The use of claim 25, wherein the water-soluble neutral salt of spermine is spermine hydrochloride and the water-soluble neutral salt of alginic acid is sodium alginate.

27. The use of claim 24, wherein the subunit component of BHV-1 comprises a BHV-1 glycoprotein, or an immunogenic fragment or derivative thereof.

-42-

28. The use of claim 27, wherein the BHV-1 glycoprotein is selected from the group consisting of gI, gII, gIII and gIV.

29. The use of claim 27, wherein the subunit component comprises BHV-1-gD.

30. The use of claim 29, wherein the subunit component consists of BHV-1-gD.

31. The use of claim 24, wherein the vaccine further comprises an adjuvant.

32. A kit for vaccinating a member of a bovine species against BHV-1, comprising a first container comprising an immunogenic amount of microcapsules comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and an immunogenic composition surrounded by the salt membrane, said microcapsules being substantially free of non-aqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of spermine and alginic acid or the interfacial reaction of a water-soluble neutral salt of spermine and a water-soluble neutral salt of alginic acid, said immunogenic composition comprising a subunit component of BHV-1 capable of inducing a protective response against BHV-1 when administered in microencapsulated form to a member of a bovine species; and a

-43-

second container comprising a diluent.

33. The kit of claim 32, wherein the water-soluble neutral salt of spermine is selected from the group consisting of halide and low molecular weight alkanolic salts, and the water-soluble neutral salt of alginic acid is selected from the group consisting of alkali metal salts, ammonium salts, and trialkanolamine salts.

34. The kit of claim 33, wherein the water-soluble neutral salt of spermine is spermine hydrochloride and the water-soluble neutral salt of alginic acid is sodium alginate.

35. The kit of claim 32, wherein the subunit component of BHV-1 comprises a BHV-1 glycoprotein, or an immunogenic fragment or derivative thereof.

36. The kit of claim 35, wherein the BHV-1 glycoprotein is selected from the group consisting of gI, gII, gIII and gIV.

37. The kit of claim 35, wherein the subunit component comprises BHV-1-gD.

38. The kit of claim 37, wherein the subunit component consists of BHV-1-gD.

-44-

39. The kit of claim 32, wherein the microcapsules are lyophilized.

40. A combination vaccine for protecting a member of a bovine species against BHV-1 and, optionally, another disease or pathological condition that afflicts a bovine species, which vaccine comprises an immunogenic amount of microcapsules comprising an anisotropic Lewis salt membrane encapsulating an aqueous or substantially aqueous core and a first immunogenic composition surrounded by the salt membrane, said microcapsules being substantially free of nonaqueous contaminants, said salt membrane comprising a precipitate resulting from the interfacial reaction of spermine and alginic acid or the interfacial reaction of a water-soluble neutral salt of spermine and a water-soluble neutral salt of alginic acid, said first immunogenic composition comprising a subunit component of BHV-1 capable of inducing a protective response against BHV-1 when administered in microencapsulated form to a member of a bovine species; an immunogenic amount of a second immunogenic composition capable of inducing a protective response against a disease or pathological condition that afflicts the bovine species; and a veterinarily acceptable carrier.

41. The combination vaccine of claim 40, wherein the water-soluble neutral salt of spermine is selected from the group consisting of halide and low molecular weight alkanolic

-45-

salts, and the water-soluble neutral salt of alginic acid is selected from the group consisting of alkali metal salts, ammonium salts, and trialkanolamine salts.

42. The combination vaccine of claim 41, wherein the water-soluble neutral salt of spermine is spermine hydrochloride and water-soluble neutral salt of alginic acid is sodium alginate.

43. The combination vaccine of claim 40, wherein the subunit component of BHV-1 comprises a BHV-1 glycoprotein, or an immunogenic fragment or derivative thereof.

44. The combination vaccine of claim 43, wherein the BHV-1 glycoprotein is selected from the group consisting of gI, gII, gIII and gIV.

45. The combination vaccine of claim 43, wherein the subunit component comprises BHV-1-gD.

46. The combination vaccine of claim 45, wherein the subunit component consists of BHV-1-gD.

47. The combination vaccine of claim 40, which further comprises an adjuvant.

-46-

48. The combination vaccine of claim 40, in which the second immunogenic composition is capable of inducing a protective response against a disease or pathological condition caused by a pathogenic organism selected from the group consisting of bovine respiratory syncytial virus, bovine viral diarrhea virus, parainfluenza virus types I, II, or III, *Leptospira* spp., *Vibrio fetus*, rotavirus, coronavirus, rabies, *Pasteurella hemolytica*, *Pasteurella multocida*, *Clostridia* spp., Tetanus toxoid, *E. coli*, and *Neospora* spp.

49. A commercial package comprising an immunogenically effective number of microcapsules according to any one of claims 1 to 7 together with instructions for use in vaccinating a member of a bovine species against BHV-1.

SMART & BIGGAR
OTTAWA, CANADA

PATENT AGENTS

FIG 1A

GGGCCGCAGC CCCGGCTGGG TATATATCCC CGACGGGCGA CTAGAGATAC ACTCGCCCCG 60
CGCGGCTGCT GCGAGCGGGC GAAC ATG CAA GGG CCG ACA TTG GCC GTG CTG 111
Met Gln Gly Pro Thr Leu Ala Val Leu
1 5
GGC GCG CTG CTC GCC GTT GCG GTG AGC TTG CCT ACA CCC GCG CCG CCG CGG 159
Gly Ala Leu Leu Ala Val Ala Val Ser Leu Pro Thr Pro Ala Pro Arg 25
10 15 20
GTG ACG GTA TAC GTC GAC CCG CCG GCG TAC CCG ATG CCG CGA TAC AAC 207
Val Thr Val Tyr Val Asp Pro Pro Ala Tyr Pro Met Pro Arg Tyr Asn 40
30
TAC ACT GAA CGC TGG CAC ACT ACC GGG CCC ATA CCG TCG CCC TTC GCA 255
Tyr Thr Glu Arg Arg Trp His Thr Thr Gly Thr Gly Pro Ile Pro Ser Pro Phe Ala 55
45 50
GAC GGC CGC GAG CAG CCC GTC GAG GTG CCG TAC GCG ACG AGC GCG GCG CGG 303
Asp Gly Arg Glu Gln Pro Val Glu Val Arg Tyr Ala Thr Ser Ala Ala 70
60 65 70
GCG TGC GAC ATG CTG GCG CTG ATC GCA GAC CCG CAG GTG GGG CGC ACG 351
Ala Cys Asp Met Leu Ala Leu Ile Ala Asp Pro Gln Val Gly Arg Thr 85
75 80 85

2/8

CTG TGG GAA GCG GTA CGC CGG CAC GCG GCG GCG TAC AAC GCC ACG GTC	399
Leu Trp Glu Ala Val Arg Arg His Ala Arg Ala Tyr Asn Ala Thr Val	105
90	
ATA TGG TAC AAG ATC GAG AGC GGG TGC GCC CGG CTG TAC TAC ATG	447
Ile Trp Tyr Lys Ile Glu Ser Gly Cys Ala Arg Pro Leu Tyr Tyr Met	120
110	
GAG TAC ACC GAG TGC GAG GAG CCC AGG AAG CAC TTT GGG TAC TGC CGC TAC	495
Glu Tyr Thr Glu Cys Glu Trp Asp Ser Phe Leu Ala Gly Tyr Cys Arg Tyr	135
125	
CGC ACA CCC CCG TTT TGG GAC AGC TTC CTG GCG GGC TTC GCC TAC CCC	543
Arg Thr Pro Pro Phe Trp Asp Ser Phe Leu Ala Gly Phe Ala Tyr Pro	150
140	
ACG GAC GAC GAG CTG GGA CTG ATT ATG GCG GCG CCC GCG CTC GTC	591
Thr Asp Asp Glu Leu Gly Leu Ile Met Ala Ala Pro Ala Arg Leu Val	165
155	
GAG GGC CAG TAC CGA CGC CTG TAC ATC GAC GGC ACG GTC GCC TAT	639
Glu Gly Gln Tyr Arg Arg Ala Leu Tyr Ile Asp Gly Thr Val Ala Tyr	185
170	
175	
180	

FIG 1A(Contd)

4/8

GAG GGC AAC GGC GGC CCC CCA GGA CCC GAA GGC GAC GGC GAG AGT CAG	1023
Glu Gly Asn Gly Gly Pro Pro Gly Pro Gly Asp Gly Glu Ser Gln	
300	310
ACC CCC GAA GCC AAC GGA GGC GCC GAG GGC GAG CCG AAA CCC GGC CCC	1071
Thr Pro Glu Ala Asn Gly Gly Ala Glu Gly Glu Pro Lys Pro Gly Pro	
315	325
AGC CCC GAC GCC GAC CGC CCC GAA GGC TGG CCG AGC CTC GAA GCC ATC	1119
Ser Pro Asp Ala Asp Arg Pro Glu Gly Trp Pro Ser Leu Glu Ala Ile	
330	340
ACG CAC CCC CCG GCC CCC GCT ACC GGC CCC GCG GCC GAC GCC GTG	1167
Thr His Pro Pro Pro Ala Thr Pro Ala Ala Pro Asp Ala Val	
350	355
CCG GTC AGC GTC GGG ATC GGC ATT GCG GCT GCG GCG ATC GCG TGC GTG	1215
Pro Val Ser Val Gly Ile Gly Ala Ala Ala Ala Ile Ala Cys Val	
365	370
	375

FIG 1B(CONTD)

FIG 1C

GCC GCC GCC GCC GCG TAC TTC GTC TAT ACG CGC CGG CGC GGT	1263
Ala Ala Ala Ala Ala Gly Ala Tyr Phe Val Tyr Thr Arg Arg Arg Gly	
	380
	385
	390
GCG GGT CCG CTG CCC AGA AAG CCA AAA AAG AAG CTG CCG GCC TTT GGC AAC	1311
Ala Gly Pro Leu Pro Arg Lys Pro Lys Lys Leu Pro Ala Phe Gly Asn	
	395
	400
	405
GTC AAC TAC AGC GCG CTG CCC GGG TGAGCGGCCT AGGCCCTCCC CCGACCGCCC	1365
Val Asn Tyr Ser Ala Leu Pro Gly	
	410
	415
CCTTTGCTCC TAGCCCCGGC TCCTGCCGAG CCGCGCGGGG	1405

5/8

FIG 4

