

## **RECONSTITUTED SURFACTANTS HAVING IMPROVED PROPERTIES**

The present invention is directed to a reconstituted surfactant comprising a lipid carrier, and a combination of a particular polypeptide analogue of the native surfactant protein SP-C with a particular polypeptide analogue of the native surfactant protein SP-B.

- 5        The invention is also directed to the pharmaceutical compositions thereof and to its use for the treatment or prophylaxis of RDS and other respiratory disorders.

### **BACKGROUND OF THE INVENTION**

10        The human lung is composed of a large number of small air sacs, called alveoli, in which gases are exchanged between the blood and the air spaces of the lungs. In healthy individuals, this exchange is mediated by the presence of a protein-containing surfactant complex that prevents the lungs from collapsing at the end of expiration.

15        Lung surfactant complex is composed primarily of lipid and contains minor amounts of various proteins. An absence of adequate levels of this complex results in malfunction of the lung. This syndrome is called Respiratory Distress Syndrome (RDS) and it commonly affects preterm infants.

20        Said syndrome is effectively treated with modified natural surfactant preparations extracted from animal lungs.

Commercially available modified surfactant preparations are, for example, Curosurf, derived from porcine lung, Infasurf, extracted from calf lung lavage and Survanta, a chemically modified natural bovine lung extract.

25        The main constituents of these surfactant preparations are phospholipids, such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine commonly known as dipalmitoyl-phosphatidylcholine (DPPC),

phosphatidylglycerol (PG) and surfactant hydrophobic proteins B and C (SP-B and SP-C).

Due to the drawbacks of the surfactant preparations from animal tissues, such as the complication of the production and sterilization processes and possible induction of immune reactions, synthetic surfactants mimicking the composition of the modified natural surfactants have been developed. Said synthetic surfactants are known as reconstituted surfactants.

However the development of clinically active reconstituted surfactants has turned out to be complicated since the native hydrophobic proteins are too big to synthesize, structurally complex and are unstable in pure form.

In order to replace said native hydrophobic proteins, some synthetic polypeptides partially corresponding to their sequences and analogs thereof have been proposed in the prior art and are for example disclosed in WO 89/06657, WO 92/22315, WO 98/49191, WO 95/32992, US 6,660,833, EP 413,957 and WO 91/18015.

WO 00/47623 disclosed synthetic polypeptides which are analogs of the native protein SP-C wherein: i) cysteine residues in position 5 and 6 have been replaced by Ser residues; ii) the Val residues of the 'center region' of SP-C have been substituted with other neutral and hydrophobic residues selected from the group consisting of Leu, Ile and norleucine (nL); iii) some of the neutral amino acid present in the 'center region' of SP-C have been replaced with bulky or polar residues selected from the group consisting of Lys, Try, Phe, Tyr, and Ornithine.

Said artificial polypeptides are characterized in that they are able of folding like the native protein SP-C and, hence, interacting properly with the surfactant lipids and do not give rise to self-oligomerisation.

WO 00/76535 generically concerns with pulmonary surfactant

preparations comprising at least one modification of the SP-B in combination with at least one modification of the SP-C protein.

In Waring AJ *et al*, (abstract presented at the Paediatric Academy Society Annual meeting held in San Francisco on April 29-May 2, 2006) a  
5 study was undertaken to examine the activity of a synthetic surfactant constituted of the SP-C-mimic SP-Cff, that is a synthetic 34-residue SP-C with phenylalanine instead of cysteine in positions 4 and 5, and the SP-B-mimic Mini-B.

However, according to the available literature, in animal studies, the  
10 treatment with reconstituted surfactants gives rise to poor lung gas volumes and grade of alveolar patency at the end of expiration, and a ventilation is required with a positive end expiratory pressure (PEEP) in order to achieve an *in vivo* activity comparable to that achieved with modified natural surfactants (Johansson J *et al* J Appl Physiol 2003, 95, 2055-2063; Davis AJ  
15 *et al* Am J Respir Crit Care Med 1998; 157, 553-559). The available reconstituted surfactant preparations are indeed not able of forming a stable phospholipidic film in the alveoli at the end of expiration.

All the aforementioned documents are silent about the problem of the alveolar patency at the end of expiration and the effect of the disclosed  
20 preparations thereof.

Therefore there is still an unmet need for a reconstituted surfactant with improved properties in terms of lung compliance.

In particular, there is a need for a reconstituted surfactant preparation which is able of guaranteeing alveolar stability, and hence maintaining  
25 alveolar patency at the end of expiration without requiring ventilation with PEEP.

It has now been found, and it is the object of the present invention, that particular analogues of the native SP-C protein, and preferably the

polypeptides of WO 00/47623, can advantageously be combined with particular analogues of the native protein SP-B in order to provide a reconstituted surfactant preparation with improved properties in terms of lung compliance, and in particular capacity to efficaciously maintain alveolar patency at the end of expiration without requiring ventilation with PEEP.

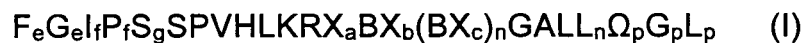
In particular it has been found that, in a model of RDS wherein the immature newborn were treated with exogenous surfactant preparations without applying PEEP, a combination of said particular analogues of the proteins SP-C and SP-B acts on the lung gas volumes which is an index of the alveolar patency at the end of expiration.

### SUMMARY OF THE INVENTION

The present invention is directed to a reconstituted surfactant comprising a lipid carrier, and a combination of a particular polypeptide analogue of the native surfactant protein SP-C with a particular polypeptide analogue of the native surfactant protein SP-B.

In particular the invention is directed to a reconstituted surfactant comprising:

- a) a lipid carrier;
- b) a polypeptide of at least 20 amino acid residues and no more than 40 amino acid residues having the sequence represented by the general formula (I)



wherein:

- 25        X                    is an amino acid residue independently selected from the group consisting of I, L, and nL;
- B                    is an amino acid residue independently selected from the group consisting of K, R, H, W, F, Y, and

Orn;

S is optionally substituted with acyl groups containing 12-22 carbon atoms, preferably 16 carbon atoms, linked to the side chain via an ester bond;

5         $\Omega$  is an amino acid selected from the group consisting of M or M oxidized on the sulfur atom, I, L, and nL;

a is an integer having a value comprised from 1 to 8;

b is an integer having a value comprised from 1 to 19;

10        c is an integer having a value independently selected from 3 to 8;

e, f, g and p are integers having a value of 0 or 1;

n is an integer having a value comprised from 0 to 3; and

15        with the condition that the  $X_aBX_b(BX_c)_n$  is a sequence having a maximum of 22 amino acids.

c) a polypeptide represented by the general formula (II):



20        wherein:

$\Delta$  is an amino acid residue independently selected from the group consisting of W and L;

$\Omega$  is an amino acid residue independently selected from the group consisting of M, I, L and nL;

25         $\Phi$  is an amino acid residue independently selected from the group consisting of R and T, preferably R; and

f is an integer having a value of 0 or 1.

The invention also includes the pharmaceutically acceptable salts of

said polypeptides and their blocked N- and/or C-terminus derivatives, e.g. *via* acetylation and amidation.

The invention is also directed to the pharmaceutical compositions thereof and to their use for the prophylaxis and/or treatment of respiratory distress syndrome (RDS) and other respiratory disorders.

The invention is further directed to the use of the polypeptides of general formula (II) for the preparation of a reconstituted surfactant to improve the alveolar patency at the end of expiration.

Finally, the invention provides a method for the prophylaxis and/or treatment of respiratory distress syndrome (RDS) and other respiratory disorders, said method comprises administering to a patient in need of such treatment a therapeutically effective amount of a reconstituted surfactant comprising a lipid carrier, a polypeptide of general formula (I), and a polypeptide of general formula (II).

## FIGURES

Figure 1 shows the sequence of human protein SP-C. The Cys residues are palmitoylated in native SP-C.

Figure 2 shows the sequence of human protein SP-B.

Figure 3 shows the results in terms of lung gas volumes (ml/kg).

Figure 4 shows the results in terms of tidal volumes (ml/kg) as a function of time/pressure.

## DEFINITIONS

The respiratory function after *in vivo* treatment with the exogenous surfactant preparations is carried out by measuring two parameters:

- i) the tidal volume which is an index of the lung compliance and
- ii) the lung gas volume which is an index of the alveolar air expansion or patency at the end of expiration, and hence of the capability of forming a stable phospholipidic film in the alveoli at the end of expiration.

In the present application, the term “reconstituted surfactant” means a lipid carrier to which polypeptide analogues of the surfactant proteins, made through recombinant technology or synthetic methods, have been added.

The term “lipid carrier” means a mixture of phospholipids and optionally further lipid components, for example neutral lipids such as triacylglycerols, free fatty acids and/or cholesterol.

The term “polypeptide analogues of the native surfactant protein SP-C”, includes polypeptides having an amino acid sequence in which, compared to the native proteins, one or more amino acids are missing or have been replaced by other amino acids, so long as the polypeptides, in a mixture with a lipid carrier, show pulmonary surfactant activity.

The term “polypeptide analogues of the native surfactant protein SP-B”, includes peptides having an amino acid sequence in which, compared to the native proteins, one or more amino acids are missing so long as the polypeptides, in a mixture with a lipid carrier, show pulmonary surfactant activity.

The term “mini-B” means a 34-residue polypeptide based on the N-terminal residues 8-25 and C-terminal residues 63-78 of the native SP-B protein whose structure was first generically disclosed in a presentation taken from the California NanoSystems Institute website. Its full sequence has been subsequently disclosed in the RCSB Protein Data Bank.

In Waring AJ *et al* J Peptide Res 2005, 66, 364-374, more information about its structure and activity was reported.

The term “variants” means polypeptides analogues of the Mini-B peptide having an amino acid sequence in which one or more amino acids have been replaced by other amino acids, so long as the peptides, in a mixture with a lipid carrier, retain the activity of Mini-B.

The amino acid sequences are shown according to the three-letter

code with the amino acid which carries the free amino group at the left end (amino terminus) and the amino acid which carries the free carboxyl group at the right end (carboxy terminus).

The term "synergistic" means that the effectiveness of the two polypeptides is more than would be expected by summing their respective individual effectiveness in a given assay.

All the amino acid residues identified herein are in the natural L-configuration and the sequences identified herein are reported according to standard abbreviations for amino acid residues as shown in the following Table of Correspondence.

#### TABLE OF CORRESPONDENCE

| <u>AMINO ACID</u> |              | <u>SYMBOL</u>     |                     |
|-------------------|--------------|-------------------|---------------------|
|                   |              | <u>One-letter</u> | <u>Three-letter</u> |
| 15                | Glycine      | G                 | Gly                 |
|                   | L-proline    | P                 | Pro                 |
|                   | L-isoleucine | I                 | Ile                 |
|                   | L-leucine    | L                 | Leu                 |
|                   | L-tyrosine   | Y                 | Tyr                 |
| 20                | L-cysteine   | C                 | Cys                 |
|                   | L-tryptophan | W                 | Trp                 |
|                   | L-alanine    | A                 | Ala                 |
|                   | L-lysine     | K                 | Lys                 |
|                   | L-arginine   | R                 | Arg                 |
| 25                | L-glutamine  | Q                 | Glu                 |
|                   | L-methionine | M                 | Met                 |
|                   | L-serine     | S                 | Ser                 |
|                   | L-valine     | V                 | Val                 |

|   |                 |   |      |
|---|-----------------|---|------|
|   | L-asparagine    | N | Asn  |
|   | L-aspartic acid | D | Asp  |
|   | L-glutamic acid | E | Gln  |
|   | L-histidine     | H | His  |
| 5 | L-threonine     | T | Thr  |
|   | L-phenylalanine | F | Phe  |
|   | L-nor-leucine   | - | nLeu |
|   | L-ornithine     | - | Orn  |

### DETAILED DESCRIPTION OF THE INVENTION

10       The present invention is directed to a reconstituted surfactant comprising a lipid carrier and a combination of particular polypeptide analogues of the native surfactant protein SP-C with particular polypeptide analogues of the native surfactant protein SP-B including the Mini-B peptide and variants thereof.

15       We have indeed found that, in a model of RDS wherein the immature newborn were treated with exogenous surfactant preparations without applying PEEP, a combination of a polypeptide of general formula (I), with a polypeptide of general formula (II) positively acts on the lung compliance.

20       In particular, the combination of the two polypeptides was found to act in a synergistic way on the lung gas volumes which is an index of the alveolar patency at the end of expiration.

25       Said result demonstrates that the reconstituted surfactant of the invention is able to stabilise the phospholipid film in the alveoli at the end of expiration in a better way than reconstituted comprising only an analogue of the protein SP-C or an analogue of the protein SP-B.

      Moreover, the claimed reconstituted surfactant preparation turned out to improve the respiratory function as expressed by the tidal volumes to an extent comparable or even slightly better than that achieved after

administration of a modified natural surfactant.

Advantageously, the analogue of the native protein SP-C is a polypeptide of at least 20 amino acid residues and no more than 40 amino acid residues, having the sequence represented by the general formula (I)

5



wherein:

- |    |                      |                                                                                                                    |
|----|----------------------|--------------------------------------------------------------------------------------------------------------------|
|    | X                    | is an amino acid residue independently selected from the group consisting of I, L, and nL;                         |
| 10 | B                    | is an amino acid residue independently selected from the group consisting of K, R, H, W, F, Y, and Orn;            |
|    | S                    | is optionally substituted with acyl groups containing 12-22 carbon atoms, preferably 16 carbon atoms,              |
| 15 |                      | linked to the side chain via an ester bond;                                                                        |
|    | $\Omega$             | is an amino acid residue selected from the group consisting of M or its oxidized on the sulfur atom, I, L, and nL; |
|    | a                    | is an integer having a value comprised from 1 to 8;                                                                |
| 20 | b                    | is an integer having a value comprised from 1 to 19;                                                               |
|    | c                    | is an integer having a value independently selected from 3 to 8;                                                   |
|    | <i>e, f, g and p</i> | are integers having a value of 0 or 1;                                                                             |
| 25 | <i>n</i>             | is an integer having a value comprised from 0 to 3;                                                                |
|    |                      | and                                                                                                                |

with the condition that the  $X_a B X_b (B X_c)_n$  is a sequence having a maximum of 22 amino acids, preferably comprised between 10 and 22 amino

acids.

Preferably, the polypeptide of general formula (I) consists of at least 30 and not more than 35 amino acids, more preferably not more than 33 amino acids.

5 In particular embodiments the polypeptides of general formula (I) consist of 30 or 33 or 35 amino acids.

Preferably, the polypeptide analogue of the SP-C protein is represented by the general formula (Ia) in which  $e$  and  $n$  are 0, and  $g$  is 1

10 
$$I_f P_i S S P V H L K R X_a B X_b G A L L \Omega_p G_p L_p \quad (Ia)$$

wherein

$X$ ,  $B$  and  $\Omega$  are as defined above;

$a$  is from 1 to 8, preferably from 1 to 3; more preferably 1;

$b$  is from 1 to 19, preferably from 5 to 15; more preferably 14;

15  $f$  and  $p$  are 0 or 1.

More preferably, the polypeptide analogue of the SP-C protein is represented by the general formula (Ib) in which  $f$  is 1

$$I P S S P V H L K R X_a B X_b G A L L \Omega_p G_p L_p \quad (Ib)$$

20 wherein:

$X$ ,  $B$ ,  $\Omega$ ,  $a$  and  $b$  are as defined above;

$p$  is 0 or 1.

Even more preferably, the polypeptide analogue of the SP-C protein is represented by the general formula (Ic)

25 
$$I P S S P V H L K R L K L L L L L L L L L L L L L L L G A L L \Omega_p G_p L_p \quad (Ic)$$

wherein:

$\Omega$  is as defined above

$p$  is 0 or 1

Examples of polypeptides of formula (Ic) are reported below:

|   |                                    |                 |
|---|------------------------------------|-----------------|
|   | IPSSPVHLKRLKLLLLLLLLLILLILGALLMGL  | (Id) (SEQ ID:1) |
| 5 | IPSSPVHLKRLKLLLLLLLLLILLILGALLIGL  | (Ie) (SEQ ID:2) |
|   | IPSSPVHLKRLKLLLLLLLLLILLILGALLLGL  | (If) (SEQ ID:3) |
|   | IPSSPVHLKRLKLLLLLLLLLILLILGALLnLGL | (Ig) (SEQ ID:4) |
|   | IPSSPVHLKRLKLLLLLLLLLILLILGALL     | (Ih) (SEQ ID:5) |

The polypeptide (Id) has been also referred to in the prior art as  
 10 SP-C33.

Most preferably, the SP-C analogue is a polypeptide selected from the group of polypeptides having the formulae (Ie), (If), (Ig) and (Ih).

In a preferred embodiment of the invention, the polypeptide of general formula (I) is the polypeptide (If) hereinafter referred to as SP-C33(Leu)<sup>31</sup>.

15 Advantageously, the analogue of the native protein SP-B consists of two portions corresponding to the N-terminal residues 8-25 and to the 63-78 C-terminal part of the native SP-B protein (referred to as Mini-B peptide) or a variant thereof.

More advantageously, the analogue of the native protein SP-B is a  
 20 polypeptide represented by the following general formula (II):



wherein:

25  $\Delta$  is an amino acid residue selected from the group consisting of W and L;

$\Omega$  is an amino acid residue independently selected from the group consisting of M, I, L, and nL;

$\Phi$  is an amino acid residue selected from the group consisting of R

and T, preferably R; and

$f$  is an integer having a value of 0 or 1.

In a particular embodiment of the invention, the SP-B analogue is a polypeptide represented by the formula (IIa)

5

CWLCRALIKRIQAMIPKGG RMLPQLVCRLVLRCS (IIa) (SEQ ID:6)

In another embodiments of the invention the SP-B analogue is selected from those having the following formulae

10 

CLLCRALIKRIQAMIPKGG RMLPQLVCRLVLRCS (IIb) (SEQ ID:7)

CWLCRALIKRIQALIPKGG RLLPQLVCRLVLRCS (IIc) (SEQ ID:8)

CLLCRALIKRIQALIPKGG RLLPQLVCRLVLRCS (IId) (SEQ ID:9)

In a preferred embodiment, the polypeptides (IIa), (IIb), (IIc) and (IId) may be in the form of disulfide linked molecule wherein the intramolecular  
15 disulfide linkage is between the two Cys residues in position 1 and 33 and/or between the two Cys residues in position 4 and 27.

The disulfide linked polypeptide (IIa) has been referred in the art to as oxidized Mini-B (ox Mini-B).

20 

The polypeptide (IIc) is hereinafter referred to as Mini-B(Lleu) and its disulfide linked form as ox Mini-B(Leu).

The polypeptides of general formulae (I) and (II) may be prepared according to synthetic methods or recombinant techniques well known to the person skilled in the art.

25 

An excellent summary of the many techniques available may be found in J.M. Steward and J.D. Young, "Solid Phase Peptide Synthesis", W.H. Freeman Co., San Francisco, 1969, and J. Meienhofer, Hormonal Proteins and Peptides", Vol.2, p. 46, Academic Press (New York), 1983 for solid phase peptide synthesis, and E. Schroder and K. Kubke, "The Peptides", Vol.

1, Academic Press (New York), 1965 for classical solution synthesis. The polypeptides of the invention can also be prepared using the solid-phase synthetic technique initially described by Merrifield, in J. Am. Chem. Soc. 85: 2149-2154 (1963). Other polypeptide synthesis techniques may be found, for example, in M. Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed., (1976) as well as in other reference works known to those skilled in the art.

Appropriate protective groups for use in such syntheses will be found in the above texts as well as in J.F.W. McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, NY (1973).

For example, the polypeptides of general formula (I) may be prepared according to the method disclosed in WO 00/47623, while the polypeptides of general formula (II) may be prepared according to the method reported in Waring AJ et al J Peptide Res 2005, 66, 364-374.

The invention also includes the pharmaceutically acceptable salts the polypeptides of general formulae (I) and (II) and their blocked N- and/or C-terminus derivatives, e.g *via* acetylation and amidation.

Pharmaceutically acceptable salts include for example, salts of hydrochloric acid, acetic acid, and trifluoroacetic acid.

The reconstituted surfactant of the invention may be prepared by mixing a solution or a suspension of the polypeptides of general formula (I) and (II) and lipids and by subsequently drying the mixture, otherwise they may be prepared by lyophilisation or spray-drying.

Preferably, the polypeptides of general formula (I) and the polypeptides of general formula (II) are present in the reconstituted surfactants of the invention in a fixed amount and quantitative ratio as a fixed combination.

The proportion of the polypeptides of general formulae (I) and (II)

relative to the reconstituted surfactant can vary. Advantageously each polypeptide may be present in an amount comprised between 0.5 and 10% based on the weight of the surfactant (w/w), preferably between 1 and 5%, most preferably between 1 and 3%.

5 In a preferred embodiment, the reconstituted surfactant comprises from 1 to 3% by weight of the polypeptide (If), from 1 to 3% by weight of the polypeptide (IIa), preferably in the oxidized form.

In another preferred embodiment the reconstituted surfactant comprises from 1 to 3% by weight of the polypeptide (If), from 1 to 3% by weight of the polypeptide (IIc), preferably in the oxidized form.

10

Advantageously, the lipid carrier comprises the phospholipids that are contained in natural pulmonary surfactant preparations, for example phosphatidylcholines (PC) such as dipalmitoylphosphatidylcholine (DPPC) and palmitoyloleoylphosphatidylcholine (POPC), and phosphatidylglycerols (PG), such as palmitoyloleoylphosphatidylglycerol (POPG) and dipalmitoylphosphatidylglycerol (DPPG).

15

Other phospholipids which can be advantageously used are phosphatidylinositols (PI), phosphatidylethanolamines (PE), phosphatidylserines and sphingomyelins (SM).

20 In a particular embodiment, the lipid carrier may comprise further components, for example neutral lipids such as triacylglycerols, free fatty acids and/or cholesterol.

Advantageously the reconstituted surfactant according to the invention comprise 90 to 99% by weight of a lipid carrier, preferably 92 to 98%, more preferably 94 to 96%, and 1 to 10% by weight of the sum of both peptides, preferably 2 to 8%, more preferably 4 to 6%.

25

In one of the embodiments of the invention the reconstituted surfactant comprises 96% by weight of a lipid carrier, 2% by weight of a polypeptide of

general formula (I) and 2% by weight of a polypeptide of general formula (II).

In a particular embodiment the lipid carrier only consists of phospholipids more preferably of a mixture of DPPC and a palmitoyloleoylphospholipid selected from POPG or a mixture thereof with  
5 POPC in weight ratios ranging from 95:5 to 50:50, preferably from 80:20 to 60:40.

The weight ratio between DPPC and POPG ranges preferably from 75:25 to 65:35, and is more preferably 68:31. In the case of DPPC:POPG:POPC mixtures, the phospholipids are preferably used in  
10 weight ratios of 60:20:20 or 68:15:16.

In another embodiment, the lipid carrier may consist of DPPC, DPPG and cholesterol.

In a preferred embodiment of the invention, the reconstituted surfactant comprises from 1 to 5% by weight of one the polypeptides of  
15 general formula (Ia), from 1 to 5% by weight of one of the polypeptides of general formula (II) and a mixture of DPPC and POPG in a weight ratio of 68:31.

The administration of the reconstituted surfactant of the invention is carried out in a manner known to the person skilled in the art, preferably by  
20 intratracheal installation (infusion or bolus) or by nebulisation.

The present invention also concerns pharmaceutical compositions comprising the reconstituted surfactant of the invention. Said compositions are advantageously administered in the form of a solution, dispersion, suspension or dry powder. Preferably said compositions comprise the  
25 reconstituted surfactant dissolved or suspended in a suitable solvent or resuspension medium.

Preferably said pharmaceutical compositions are supplied as suspension in a buffered physiological saline aqueous solution in single-use

glass vials. Advantageously the reconstituted surfactant concentration (expressed as phospholipid content) is in the range of from about 2 to about 160 mg of surfactant per ml, preferably between 10 and 100 mg/ml, more preferably between 20 and 80 mg/ml.

- 5            Said compositions may further comprise electrolytes, such as calcium, magnesium and/or sodium salts, preferably calcium chloride.

          The pharmaceutical compositions according to the invention are suitable for the prophylaxis and/or treatment of respiratory distress syndrome (RDS) in prematurely born babies or other diseases related to a surfactant-  
10    deficiency or dysfunction including RDS in adults (ARDS), meconium aspiration syndrome (MAS), and bronchopulmonary dysplasia (BPD).

          They may also be useful for the prophylaxis and/or treatment of other respiratory disorders such as pneumonia, bronchitis, COPD (chronic obstructive pulmonary disease), asthma, and cystic fibrosis as well as for the  
15    treatment of serous otitis media (glue ear).

          The following examples illustrate the invention in more details.

### **EXAMPLES**

#### **Example 1 - Synthesis and purification of the polypeptides SP-C33(Leu)<sup>31</sup> and SP-C33.**

20            The polypeptide SP-C33(Leu)<sup>31</sup> was prepared by standard SPPS (Solid Phase Peptide Synthesis) methods based on Fmoc chemistry and consecutive TFA-cleavages.

          In total 186.0 g of crude SP-C33(Leu)<sup>31</sup> were obtained.

          The polypeptide was purified by subjecting the material to counter  
25    current distribution (CCD) using H<sub>2</sub>O/n-BuOH/AcOEt/AcOH 4:1:4:1 as biphasic solvent system.

          This purification yielded 78.9 g of SP-C33(Leu)<sup>31</sup> with a purity of > 60%.

The final purification was carried out by preparative HPLC using PLRP-S as stationary phase in a steel column of 50 x 300 mm applying a linear gradient of 25% B to 100% B in 75 minutes. The mobile phase consisted of buffer A = 0.1% TFA in ACN/H<sub>2</sub>O 1:4 and buffer B = 0.1% TFA  
5 in IPA.

The purified polypeptide was dissolved in 90% AcOH and passed through a column packed with Dowex ion exchange resin (acetate form) to furnish, after lyophilisation, 5.8 g (= 5.4%) of the final product as the acetate.

The polypeptide SP-C33 was prepared in an analogous manner.

10 **Legend**

|           |                              |
|-----------|------------------------------|
| ACN       | Acetonitrile                 |
| AcOEt     | Ethyl acetate                |
| AcOH      | Acetic acid                  |
| Boc       | t-Butyloxycarbonyl           |
| 15 n-BuOH | n-Butanol                    |
| Fmoc      | 9-Fluorenylmethyloxycarbonyl |
| IPA       | Isopropyl alcohol            |
| TFA       | Trifluoroacetic acid         |

20 **Example 2 - Synthesis and purification of the polypeptides ox Mini-B(Leu) and ox-Mini-B.**

The polypeptide ox-Mini-B(Leu) was prepared by standard SPPS (Solid Phase Peptide Synthesis) methods based on Fmoc chemistry and consecutive TFA-cleavages.

25 The crude polypeptide was purified with preparative HPLC using a TFA system and isolated by lyophilisation.

Air oxidation of the purified peptide yielded the monocyclic sequence with the disulfide bond between Cys<sup>1</sup> and Cys<sup>33</sup>. The monocyclic peptide was purified with preparative HPLC using a TFA system and isolated by

lyophilisation.

The second disulfide bridge between Cys<sup>4</sup> and Cys<sup>27</sup> was formed using iodine.

After oxidation, the product was purified with preparative HPLC using a TFA system and isolated by lyophilisation. 1.12 g (= 1.7%) of the final compound were isolated with a purity of >89%.

The polypeptide ox Mini-B was prepared in an analogous manner.

**Example 3 - In vivo experiments with a reconstituted surfactant based on ox Mini-B and SP-C33**

The surfactant preparations were assayed in premature newborn rabbits, obtained by hysterectomy at the gestational age of 27 days. The experiments were performed without applying a positive end expiratory pressure (PEEP).

As SP-C analogue, the polypeptide referred to as SP-C33 was used which was prepared according to Example 1.

As analogue of the protein SP-B, oxidized Mini-B (ox Mini-B) was used which was prepared according to Example 2.

The animals were treated at birth with reconstituted surfactant preparations containing, as lipid carrier, the phospholipid mixture DPPC:POPG in the ratio 68:31 w/w. The phospholipids were mixed with 2 or 4% w/w SPC-33, 2% w/w ox Mini-B or 2% w/w SPC-33 plus 2% w/w ox Mini-B.

Animals receiving the same dose of Curosurf® served as positive controls and non-treated littermates as negative controls.

All surfactant preparations were administered at a concentration of 80 mg/ml and at a standard dose of 2.5 ml/kg.

The immature newborn rabbits were ventilated in parallel with a standardized sequence of peak insufflation pressures. To open up the lungs,

pressure was first set at 35 cm H<sub>2</sub>O for 1 min. After this recruitment manoeuvre, pressure was lowered to 25 cm H<sub>2</sub>O for 15 min and further on to 20 and 15 cm H<sub>2</sub>O. Finally, pressure was raised again to 25 cm H<sub>2</sub>O for 5 min, after which the lungs were ventilated for additional 5 min with nitrogen  
5 and then excised for gas volume measurements.

Both lung gas volumes and tidal volumes expressed as ml/kg were measured and the results, given as median values, are reported in Figures 3 and 4, respectively.

From Fig. 3 it can be appreciated that animals treated with the reconstituted surfactant preparation containing 2% w/w ox Mini-B had lower  
10 lung gas volumes than animals that received 2% w/w SP-C33 (about 2 ml/kg vs about 4 ml/kg).

Addition of 2% w/w ox Mini-B to SP-C33 surfactant produced a significantly greater increment in lung gas volumes than either peptide alone  
15 (8 ml/kg vs about 6 ml/kg).

As shown in Fig.4, a similar trend was observed for the tidal volumes and, after administration of the peptides in combination an improvement comparable or even slightly better than that achieved after administration of Curosurf® was observed.

20 It has also been found that the increase of SP-C33 content from 2 to 4% w/w did not increase lung gas volumes indicating that the stabilising effect of the phospholipid film in the alveoli at the end of expiration is due to the addition of ox Mini-B.

Said result demonstrates that the reconstituted surfactant of the invention is able to stabilise the phospholipid film in the alveoli at the end of  
25 expiration in a better way than reconstituted comprising only an analogue of the protein SP-C or an analogue of the protein SP-B.

Moreover, the claimed reconstituted surfactant preparation turned out

to improve the respiratory function as expressed by the tidal volumes to an extent comparable or even slightly better than that achieved after administration of a modified natural surfactant.

**CLAIMS**

1. A reconstituted surfactant comprising a lipid carrier and a combination of a polypeptide analogue of the native surfactant protein SP-C with a  
5 polypeptide analogue of the native surfactant protein SP-B

wherein said polypeptide analogue of the native surfactant protein SP-C is a polypeptide of at least 20 amino acid residues and no more than 40 amino acid residues having the sequence represented by the general formula  
(I)

10



wherein:

- |                             |                                                                                                                                                   |
|-----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| X                           | is an amino acid residue independently selected from the group consisting of I, L, and nL;                                                        |
| 15      B                   | is an amino acid residue independently selected from the group consisting of K, W, F, Y, and Orn;                                                 |
| S                           | is optionally substituted with acyl groups containing 12-22 carbon atoms, preferably 16 carbon atoms, linked to the side chain via an ester bond; |
| 20 $\Omega$                 | is an amino acid residue selected from the group consisting of M or its oxidized on the sulfur atom, I, L, and nL;                                |
| a                           | is an integer having a value comprised from 1 to 8;                                                                                               |
| b                           | is an integer having a value comprised from 1 to 19;                                                                                              |
| 25      c                   | is an integer having a value independently selected from 3 to 8;                                                                                  |
| <i>e, f, g</i> and <i>p</i> | are integers having a value of 0 or 1;                                                                                                            |
| <i>n</i>                    | is an integer having a value comprised from 0 to 3;                                                                                               |

with the condition that the  $X_aBX_b(BX_c)_n$  is a sequence having a maximum of 22 amino acids; and

wherein said polypeptide analogue of the native surfactant protein SP-B is a polypeptide of general formula (II):

5



wherein:

$\Delta$  is an amino acid residue selected from the group consisting of W and L;

10  $\Omega$  is an amino acid residue independently selected from the group consisting of M, I, L, and nL;

$\Phi$  is an amino acid residue selected from the group consisting of R and T; and

$f$  is an integer having a value of 0 or 1.

15 2. A reconstituted surfactant according to claim 1 wherein  $\Phi$  is R.

3. A reconstituted surfactant according to claim 1 or 2 wherein the analogue of the native surfactant protein SP-C and the analogue of the native surfactant protein SP-B are present in a fixed combination.

4. A reconstituted surfactant composition according to any one of claims 1 to 3 wherein the polypeptide (II) is in the form of disulfide linked molecule with the intramolecular disulfide linkage between the two Cys residues in position 1 and 33 and/or between the two Cys residues in position 4 and 27.

5. A reconstituted surfactant composition according to any one of claims 1 to 3 wherein the analogue of the native surfactant protein SP-B is  
25 represented by the general formula (IIa)



6. A reconstituted surfactant according to claim 5 wherein the analogue

of the native surfactant protein SP-C is represented by the general formula (Ia)



5            wherein

X, B and  $\Omega$  are as defined above;

a    is from 1 to 8, preferably from 1 to 3; more preferably 1;

b    is from 1 to 19, preferably from 5 to 15; more preferably 14; and

f and p are 0 or 1.

10    7.    A reconstituted surfactant composition according to claim 6 wherein the analogue of the native surfactant protein SP-C is represented by the general formula (Ib)



15            wherein:

X, B,  $\Omega$ , a and b are as defined above; and

p is 0 or 1.

8.    A reconstituted surfactant composition according to claim 7 wherein the analogue of the native surfactant protein SP-C is represented by the  
20    general formula (Ic)



wherein:

$\Omega$     is as defined above; and

25    p    is 0 or 1.

9.    A reconstituted surfactant composition according to claim 8 wherein the analogue of the native surfactant protein SP-C is selected from the group consisting of:

IPSSPVHLKRLKLLLLLLLLLILLILGALLMGL (Id)

IPSSPVHLKRLKLLLLLLLLLILLILGALLIGL (Ie)

IPSSPVHLKRLKLLLLLLLLLILLILGALLLGL (If)

IPSSPVHLKRLKLLLLLLLLLILLILGALLnLGL (Ig)

5 IPSSPVHLKRLKLLLLLLLLLILLILGALL (Ih)

10. A reconstituted surfactant according to claim 1 wherein the polypeptide analogue of the native surfactant protein SP-C is

IPSSPVHLKRLKLLLLLLLLLILLILGALLLGL (If)

10 and the polypeptide analogue of the native surfactant protein SP-B is

CWLCRALIKRIQAMIPKGGRMLPQLVCRLVLRCS (IIa)

11. A reconstituted surfactant according to claim 1 wherein the polypeptide analogue of the native surfactant protein SP-C is

15

IPSSPVHLKRLKLLLLLLLLLILLILGALLLGL (If)

and the polypeptide analogue of the native surfactant protein SP-B is

CWLCRALIKRIQALIPKGGRLLPQLVCRLVLRCS (IIc)

20 12. A reconstituted surfactant according to any one of the preceding claims wherein the lipid carrier comprises a mixture of phospholipids.

13. A reconstituted surfactant according to claim 12 wherein the phospholipid mixture consists of dipalmitoyl phosphatidylcholine (DPPC) and a palmitoyl oleoyl phospholipid selected from palmitoyl oleoyl phosphatidylglycerol (POPG) or a mixture thereof with palmitoyl oleoyl phosphatidylcholine (POPC) in weight ratios ranging from 95:5 to 50:50.

25 14. A reconstituted surfactant according to claim 13 wherein the phospholipid mixture consists of DPPC and POPG in a weight ratio of 68:31.

15. A pharmaceutical composition comprising the reconstituted surfactant according to any one of claims 1 to 14.
16. A pharmaceutical composition according to claim 15 in the form of a solution, dispersion, suspension or dry powder.
- 5 17. A pharmaceutical composition according to claim 16 in the form of aqueous suspension.
18. A pharmaceutical composition according to claim 17 comprising the reconstituted surfactant in a concentration comprised between 2 and 160 mg/ml.
- 10 19. A pharmaceutical composition according to claim 18 wherein the concentration of the reconstituted surfactant is comprised between 20 and 80 mg/ml.
20. Use of a reconstituted surfactant according to any one of claims 1 to 14 for the treatment or prophylaxis of respiratory distress syndrome (RDS) in
- 15 prematurely born babies or in the treatment or prophylaxis of other diseases related to a surfactant-deficiency or dysfunction.
21. The use according to claim 20 wherein diseases related to a surfactant-deficiency or dysfunction include RDS in adults (ARDS) meconium aspiration syndrome (MAS), and bronchopulmonary dysplasia (BPD).