The use of nanocells in culture medium final products

The present invention relates to the use of nanocells in culture medium final products, to culture medium final products containing these nanocells, as well as to various uses of these culture medium final products.

Culture medum final products in the context of the present application are e.g. media for cultivating and examining cells, fungi, bacteria, viruses, bacteriophages, insects and plants, media for in vitro or ex vivo cultivation and examination of organs and tissues, media for freezing cells, fungi, bacteria, viruses, bacteriophages, insects, plants, organs and tissues, embryo-transfer-freezing media, media for therapy (adoptive immune therapy, cancer treatment) media for the perfusion of organs such as e.g. kidney or liver, media for the preservation of organs up to their transplantation as well as supplements for the above media.

The object of the invention is to work lipophilic, i.e. slightly water soluble or water insoluble substances into culture medium final products in a manner such that cells, fungi, bacteria, viruses, bacteriophages, insects, plants, tissue, organs, among other things are neither impeded in their growth nor in their biological functions.

The addition of lipophilic substances to culture media has up to now not been solved in a satisfactory manner. Often for this organic solvents such as ethanol, THF or DMSO are applied. The disadvantage with this lies in that the dissolved substances on addition to the medium, as a consequence of the thinning of the solvent may again precipitate. Precipitated hydrophobic substances adher often to the walls of the culture vessel and thus are no

longer available to the cells. Furthermore the usually applied concentrations of solvents of 0.1% already show negative effects on cells and other biological systems contained in the culture.

Apart from organic solvents, nowadays for incorporating lipophilic substances also vehicles such as micells, mixed micells, liposomes or finely emulsified emulsions are applied. Although physiologically more favourable than organic solvents, these vehicles too, in particular in high concentration, are often toxic to cells, fungi, bacteria, viruses, bacteriophages, insects, plants, tissue and organs or influence the system contained in the culture in its functioning. The functional influencing of the biological systems contained in the culture renders impossible or makes difficult the interpretation of pharmacological, toxological, metabolic and regulatoric examinations. The low loading capacity with hydrophobic substances as well as the insufficient stability in certain culture media are further reasons which may limit the application of these vehicles.

A further commonly used solutising agent is methyl-beta-cyclodextrin. Methyl-beta-cylcodextrin has however the decisive disadvantage that it render the cell membrane leaky and thus as could be repeatedly ascertained, deregulates the calcium flux; i.e. there occurs an influx of calcium into the cells with all subsequent processes activated by calcium.

It is also an object of the invention to work lipophilic compounds into culture medium final products, which are specially suitable for organ preservation up to their transplantation. Various lipid-soluble compounds are suitable for protecting cells and tissue from temporary ischemia, but also very particulary also from a re-occuring reperfusion. Such a situation results when organs are removed, temporarily preserved and later again implanted. Here

firstly there occurs a ischemic phase which after the recirculation with blood is followed by a so-called reperfusion phase with a changed oxygen transfer situation. This so-called ischemia/reperfusion, by way of the breakdown of ATP during the ischemia leads to an activation of enzyme systems which in dependence on the availability of oxygen then metabolise the breakdown products of the ATP, wherein considerable quantities of reactive oxygen compounds arise. In several studies it could be shown that various lipid-soluble antioxidants such as ß-carotin, vitamin E, but also the water-soluble vitamin C are suitable for supressing the formations of such reactive oxygen compounds including their resulting products, occuring with the ischemia/reperfusion syndrome. The perfusion media used with this were either fat solutions, or however donor organs were enriched before removal by way of oral intake of vitamins. Both solutions are unsuitable for an organ preservation. Up to now there is no other way to bring fat-soluble antioxidants into such preservation or conservation solutions.

Surprisingly it has been found that with so-called nanocells, containing

(a) a membrane-forming molecule,
(b) a coemulsifier and
(c) a lipophilic constituent,

lipohilic, i.e. water insoluble or slightly water soluble substances may be brought into culture medium final products, so that with this the cells, fungi, bacteria, viruses, bacteriophages, insects, plants or tissue contained in the culture are neither negatively affected in their growth nor in their functions. The particular advantage of this nanocell thus lies in the fact that it represents an extremely physiological application

form which in contrast to all other solutising agents of fat-soluble compounds are also easily applicable under in vivo-conditions. The fact that endothelium cells, but also other cellular compartments such as e.g. fibroblasts or intestinal mucosa cells take up vitamins in this form well, also permits a use for the application of vitamins into the buccale Mucosa.

Surprisingly with the help of nanocell technology one succeeded in bringing lipophilic compounds such as e.g. antioxidants into perfusion solutions which are suitable for being used as organ conserving solutions. In vitro and in vivo studies present up to now, which were carried out with such antioxidant formulations, show that in this manner there indeed occurs an enrichment of the anti-oxidative vitamins in the critical cell compartments, particularly in the endothelium cells. This enrichment was not expected since the cells normally may only be supplied by the mechanism of lipo-protein-bonded antioxidants. With the present experimental attempts it could thus be shown that the infusion of antioxidative vitamins via the mechanism of nanocell technology leads to a first-pass-effect in the organ, i.e. in the critical cells of the perfused organ. With this however the organ with the help of the thus applied antioxidants is protected in a physiological manner so that the application of antioxidants in this form is suitable for improving the organ preservation and conservation.

Further advantages of the nanocells are their high loading capacity with lipophilic substances as well as the good stability in culture medium final products.

The subject-matter of the present invention is therefore the use of nanocells containing as a preparative composition

(a) a membrane-forming molecule
(b) a co-emulsifier
(c) a lipophilic component

in culture medium final products.

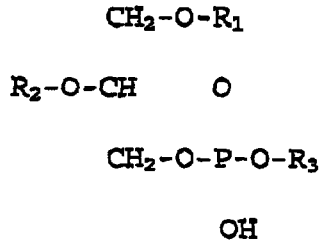
Preferably the nanocell contains

(a) as membrane-forming molecules, substances which are suitable for forming two-layer systems (so-called bilayers),
(b) as co-emulsifiers, substances which preferably form O/W structures and
(c) as a lipophilic component, a lipophilic substance usual for a culture medium.

Preferably the nanocell contains as component (a) a phospholipid, a hydrated or partly hydrated phospholipid, a lysophospholipid or mixtures of these compounds.

Very particularly preferred with this is a phospholipid of the formula:

(1)



wherein

R_1 means C_{10}-C_{20}-acyl;
R_2 hydrogen or C_{10}-C_{20}-acyl;

R_3 hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl, C_1-C_5-alkyl which is non-substituted or substituted by one or more carboxy groups, hydroxy groups or amino groups; which means inositol or glyceryl groups, or salts of these compounds.

C_{10}-C_{20}-acyl is preferably a straight-chained C_{10}-C_{20}-alkanoyl with an even number of C-atoms and straight-chained C_{10}-C_{20}-alkenoyl with one or more double bonds and an even number of C-atoms.

Straight-chained C_{10}-C_{20}-alkanoyl with an even number of C-atoms are for example n-dodecanoyl, n-tetradecanoyl, n-hexadecanoyl or n-octodecanoyl.

Straight-chained C_{10}-C_{20}-alkenoyl with one or more double bonds and an even number of C-atoms are for example 6-cis- or -6-trans-, 9-cis- or 9-trans- dodecenoyl, -tetradecanoyl, - hexadecenoyl, - octadecenoyl or -icosenoyl, in particular 9-cis-octa-decenoyl (oleoyl), further 9, 12-cis-octadecadienoyl or 9,12,15-cis-octadecatrienoyl.

A phospholipid of the formula (1), wherein R_3 means 2-trimethylamino-1-ethyl, is indicated with by trivial name lecithin and a phospholipid of the formula (1), wherein R_3 means 2-amino-1-ethyl, is indicated by the trivial name kephalin. Suitable are for example naturally occuring kephalin or lecithin, e.g. kephalin or lecithin from soya beans or hens eggs with different or identical acyl groups or mixtures thereof.

The phospholipid of the formula (1) may however also be of a synthetic origin. Under the term synthetic phospholipid one defines phospholipids which with respect to R_1 and R_2 have a uniform composition. Such synthetic phospholipids are preferably the lecithins and kephalins described later, whose acyl groups R_1

and R_2 have a defined structure and are derived from a defined fatty acid with a degree of purity higher than approx. 95%. R_1 and R_2 may be equal or different and unsaturated or saturated. Preferably R_1 is saturated, e.g. n-hexadecanoyl, and R_2 unsaturated, e.g. 9-cis-octadecenoyl (oleoyl).

The term "naturally occurring" phospholipid defines phospholipids which with respect to R_1 and R_2 have no uniform composition. Such natural phospholipids are likewise lecithins and kephalins whose acyl groups R_1 and R_2 are derived from naturally occuring fatty acid mixtures.

The demand „esentially pure" phospholipid of the formula (1) defines a degree of purity of more than 90% by weight, preferably more than 95% by weight of the phospholipid of the formula (1), which is able to be proven by way of suitable detecting methods, e.g. by paper-chromatography or by thin-layer chromatography, by HPLC or by enzymatic colour tests.

In a phospholipid of the formula (1) R_3 means C_1-C_4-alkyl, for example methyl or ethyl. The meaning methyl is preferred.

R_3 with the meanings C_1-C_5-alkyl substituted by one or more carboxy groups, hydroxy groups or amino groups are for example 2-hydroxyethyl, 2,3-dihydroxy-n-propyl, carboxymethyl, 1 or 2 carboxyethyl, dicarboxymethyl, 2-carboxy-2-hydroxyethyl or 3-carboxy-2,3-dihydroxy-n-propyl or 2-amino-2-carboxy-n-propyl, preferably 2-amino-2-carboxyethyl.

Phospholipids of the formula (1) with these groups may be present in the form of salts, e.g. as sodium or potassium salt.

Phospholipids of the formula (1), wherein R_3 means the inositol or the glyceryl group, are known under the descriptions phosphatidyl inositol and phosphatidyl glycerol.

For the acyl residue in the phospholipids of the formula (1) the descriptions indicated in brackets are also usual: 9-cis-dodecenoyl (lauroleoyl), 9-cis-tetradecenoyl (myristoleoyl), 9-cis-hexadecenoyl (palmitoleoyl), 6-cis-octadecenoyl (petroseloyl), 6-trans-octadecenoyl (petroselaidoyl), 9,12-cis-octadecenoyl (oleoyl), 9-trans-octadecenoyl (elaidoyl), 9,12-cis-octadecadienoyl (linoleoyl), 9,12,15-cis-octadecatrienoyl (linolenoyl), 11-cis-octadecenoyl (vaccenoyl), 9-cis-icosenoyl (gadoleoyl), 5,8,11,14-cis-eicosatetraenoyl (arachidonoyl), n-dodecanoyl (lauroyl), n-tetradecanoyl (myristoyl), n-hexadeconoyl (palmitoyl), n-octadecanoyl (stearoyl), n-icosanoyl (arachidoyl), n-docosanoyl (behenoyl), n-tetracosanoyl (lignoceroyl).

A salt of the phospholipids of the formula (1) is acceptable. Salts are defined by the existence of salt-forming groups in the substituent R_3 as well as by the free hydroxy group of phosphorus. Possible is likewise the formation of inner salts. Preferred are free alkali metal salts, in particular sodium salts.

In a particularly preferred embodiment form one uses purified lecithin from soja beans of the quality LIPOID S 100 or S 75 or a lecithin defined in the monography of the USP23 / NF 18.

The component (a) is preferably applied in a concentration of approx. 0.1 to 30% by weight with respect to the total weight of the components (a), (b), and (c).

As component (b) preferably an emulsifier or a mixture of emulsifiers is used, which preferably forms O/W structures.

Specially preferred emulsifiers are:

- alkali salts, ammonium salts and amine salts of fatty acids. Examples for such salts are lithium salts, sodium salts, potassium salts, ammonium salts, triethylamine salts, ethanolamine salts, diethanolamine salts or triethanolamine salts. In particular the sodium salts, potassium salts or ammonium ($NR_1R_2R_3$) salts are used, wherein R_1, R_2, R_3 independently of one another mean hydrogen, C_1-C_4-alkyl or C_1-C_4-hydroxyalkyl;
- saturated and unsaturated alkyl sulphates such as e.g. sodium docecyl sulphate and alkan sulphonates such as e.g. sodium docecane sulphonate,
- salts of the bile acids such as e.g. sodium cholate, sodium glyocholat and sodium taurocholate;
- invert soaps (Quats) such as e.g. zetyl-pyridinium chloride;
- partial fatty acid esters of sorbitane such as e.g. sorbitane monolaurate;
- sugar esters such as e.g. sucrose monolaurate;
- alkyl glucosides, such as e.g. n-octyl glucoside or n-dodecyl glucoside;
- alkyl maltoside such as e.g. n-dodecyl maltoside;
- fatty acid partial glycerides such as e.g. lauric acid monoglyceride;
- C_8-C_{18}-betaine, C_8-C_{24}-alkylamido-C_1-C_4-alkenyl betaine and C_8-C_{18}-sulphobetaine;
- fatty alcohol phosphoric acid ester,
- polyglycerine esters of fatty acids;
- propylene glycol esters of fatty acids;
- lactic acid esters of fatty acid such as e.g. sodium stearoyl-lactyl-2-lactate;
- proteins such as e.g. casein.

Emulsifiers of the polyoxyethylene type are very particularly preferred. Examples of such emulsifiers are

- polyethoxylised sorbitane fatty acid ester such as e.g. polysorbate 80;
- polyethoxylised vitamin E derivatives such as e.g. vitamin E polyethylene glycol 1000 succinate;
- polyoxyethyleneglycolised natural or hydrated vegetable oils such as e.g. polyoxyethylene-glycolised natural or hydrated castor oils;
- polyethoxylised fatty acid partial glycerides such as e.g. diethyleneglycol monostearate;
- polyethoxylised fatty alcohols such as e.g. oleth-20,
- polyethoxylised fatty acid such as e.g. polyoxyl 20 stearate,
- polyethoxylised lanolin and its derivatives such as e.g. laneth-20,
- polyethoxylised carbohydrates;
- blockcopolymerisates of ethylene oxide and propylene oxide, such as e.g. Poloxamer 188.

The component (b) is present in the nanocell used according to the invention in a concentration of approx. 1 to approx. 50% by weight with respect to the total weight of the components (a), (b) and (c).

The component (c) is preferably a natural, a synthetic or a partial synthetic di- or triglyceride, a lipophilic substance common for culture media or mixtures of these substances.

For culture medium final products suitable substances are e.g. essential substances enhancing the growth of cells, fungi, bacteria, viruses, bacteriophages, insects, plants, tissue, amongst others, such as vitamins, amino acids, peptides, natural or recombinant proteins, carbohydrates, lipids, nucleic acids,

ribonucleosides, deoxyribonucleosides, anorganic salts and trace elements; further natural, semi-synthetic or synthetic active ingredients such as antioxidants, antibiotics, antimyotics, hormones; substance mixtures for screenings and markers for examining cell-biological functions.

Examples of vitamins are vitamin A, vitamin B1, vitamin B2, vitamin B6, nicotine amide, pantothenic acid, vitamin B12, vitamin B15, folic acid, biotin, vitamin C, vitamin D, vitamin E, vitamin F, vitamin K and vitamin P.

Examples of amino acids and peptides are alanine, valine, leucine, isoleucine, proline, phenyl alanine, tryptophane, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, glycyl-L-glutamine, L-alanyl-L-glutamine and hydroxy-L-proline.

Examples of natural and recombinant proteins are growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), acidic and basic fibroblast growth factor (aFGF and bFGF), insulin-like growth factor I and II (IGF-I and IGF-II), nerve growth factor (NGF), platelet-derived growth factors (PDGFs), stem cell factors and transforming growth factors, chemotactical factors such as macrophages/monocyte chemotactical and activating factor (MCAF); colony-stimulating factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte-colony-stimulating factor (G-CSF), interferone and interleucine; cell adhesion and extra-cellular matrix proteins such as collagens, fibronectines, integrines, laminines, merosins, proteoglycanes, RGD-adhesion peptides, tenascine, thrombospondine and vitronectine and enzymes such as collagenase, dipase and

trypsine. Further examples of proteins are insulin, albumin and transferrin.

Examples of carbohydrates are glucose, galactose, fructose, sucrose, maltose, ribose, deoxyribose, trehalose, tryptose and yeastolate.

Examples of lipids are saturated and unsaturated fatty acids as well as derivatives thereof. Examples of saturated fatty acids are myristic acid, palmitic acid, stearic acid. Examples of unsaturated fatty acids are omega-9-fatty acids such as e.g. oeleic acid, omega-6-fatty acids such as e.g. linoleic acid, gamma-linolenic acid, dihommo-gamma-linolenic acid and arachidonic acid and omega-3-fatty acids such as e.g. alpha linolenic acid, stearidonic acid, eicosapentanoic acid and docosahexanoic acid. Further examples of lipids are cholesterol and derivatives thereof.

Examples of nucleic acids, ribonucleosides and deoxyribonucleosides are adenine, guanine, thymidine, uracil, adenosine, guanosine, cytidine, uridine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, 2'-deoxythymidine, 5-methyl-deoxycytidine and 5-methylcytosine.

Examples of anorganic salts and trace elements are $CACl_2$, KCl. $MgSO_4$, NaCl, $NaHCO_3$, NaH_2PO_4, $Fe(NO_3)$, $FeSO_4$, KH_2PO_4, MgC_{12}, $(NH_4(MO_7O_{24})$, $CuSO_4$, KNO_3, $ZnSO_4$, $NiCl_2$, Na_2Si0_3, H_2SeO_3, $MnSO_4$ and $Ca(NO_3)_2$.

Examples of antioxidants are vitamin E and derivatives, tocotrienols, vitamin A and derivatives, vitamin C, beta-carotine, carotinoids, ubiquinones such as coenzyme Q10, flavonoids,

isoflavonoids, polyphenols, phytooestrogenes, lycopenes, luteins, lipon acids, glutathiones.

Examples of antibiotics and antimyotics are tylocine, gentamycine-sulphate, kanamycine sulphate, neomycine sulphate, nystatin, polymixin B-sulphate, streptomycine sulphate, actino-mycine D, penicillin G, ampicillin, carbenicillin, amphotericin B and mycophenolic acid.

Examples of hormones are hydrocortisone, progesterone, dexamethasone and triamzinolon.

Examples for substance mixtures for screenings are plant or animal extracts with bioactive ingredient substances.

Examples of markers for examining cell-biological functions are flourescence colourings, immunhistochemical markers and vital markers.

Likewise examples of substances for culture media are coenzymes such as diphosphopyridine nucleotide (DPN), flavine-adenine-dinucleotide (FAD), triphosphopyridine-nucleotide (TPN) and uridine triphosphate (UTP), coenzyme A, cocarboxylase; peptones such as meat peptones, casein peptone, soja peptone, bacto peptone and lactalbuminhydrolysate as well as extracts such as yeast extract, bovine hypophysene extract, chicken-embryo extract or beef extract.

Further examples of substance for culture media are putrescine, phenol red, fumaric acid, malic acid, alpha-ketoglutaric acid, succinic acid, lactic acid, DL-68-thiocitic acid, para-aminobenzoic acid, HEPES, Ca-lactate, Na-succinate, Na-pyruvate, Na-glucuronate, Na-acetate, Na-mucate, D-glucurone-lactone,

aminopterine, hypoxanthine, xanthine, choline chloride, choline bitartrate, taurine and salmine sulphate.

The component (c) is present in the nanocell applied according to the invention in a preferred concentration of 0.1 to 70% by weight with respect to the total weight of the components (a), (b) and (c).

Where appropriate the nanocell applied according to the invention contains as a facultative component (d) a solutising agent, preferably C_2-C_8 alcohol such as e.g. ethanol or propylene glycol.

In a few special cases the nanocell applied according to the inventions contains only the components (b) and (c).

A nanocell composition with the components (a), (b) and (c) is distinguished with the dispersion in an aqueous medium by favourable phase properties. Thus with a present opalescence and transparency in counterlight it can only be recognised with an extremely slight milky cloudiness that the dispersion still has physical differences with respect to the ideal condition of a purely molecular solution. Electron microscope imagings show that a population of more than 98% in a normal distribution is present as a suspension of particles (nanoparticles) with a particle size smaller than approx. 100 nm (nanodispersion), typically smaller than approx. 50 nm. These differences with respect to a pure solution however on account of the particularly good homogeneity properties of the dispersion may be tolerated, which for example may be shown by a surprisingly high storage stability, i.e. no demixing after a storage for several months at temperatures up to room temperature (by extrapolation, the stability to be expected is longer than two years).

For manufacturing the nanocells the two components (b) and (c) are mixed to a homogeneous fluid phase, where appropriate through heating. In this phase the component (a) is dissolved, where appropriate with the help of a solubiliser such as ethanol. From this there results a clear liquid, the so-called nanocell prephase. This is added through stirring to the aqueous phase, which where appropriate contains water soluble substances. The stirring is effected with usual stirring apparatus, such as e.g. propeller stirring devices, slant-blade stirring devices or magnet stirring devices. By way of the particular selection of the components (a), (b) and (c) there arise at the same time direct ultra-fine, mono-dispersed nanocells. With this one may do away with a homogenisation by way of nozzle homogenisers, rotor-stator homogenisers or ultrasound homogenisers, which produce high shear forces and cavitation forces.

The addition of the nanocell prephase to the aqueous phase is usually carried out at a temperature < 50° C.

The nanocells characterised by the described manufacturing method have an average particle diameter of smaller than 100 nm, typically smaller than 50 nm. The particle distribution is mono-dispersed and obeys a normal distribution. Laser light scattering measurements and electron microscope examinations (Cryo-TEM) confirm the very small size and excellent homogeneity of the nanocells.

The nanocells according to the invention are used in culture medium final products.

These culture medium final products are preferably liquid or semi-solid but however may also be solid.

Examples of culture medium final products are animal serums such as foetal serum, serum of new-born calves, calf serum. Serum from other animal types, bovine serous fluids, synthetic culture media such as serum-free/serum-reduced media, minus media for radioactive incorporative studies, media for molecular and zytogenetics, selection media, media for the plaque assay, media for detecting mycoplasms, media for freezing, media for the transfer of tissue, organs and embryos, media for therapy, media for the perfusion of organs, media for preserving organs up to their transplantation, media for pharmacological, toxicological and metabolic studies, media for uptake and transport studies, media for examining functional and regulational mechanisms, supplements such as enzyme, amino acid and vitamin solutions; lipid concentrates, growth-stimulating additions, growth factors containing serum replacement products, vitamins, binding proteins, hormones, adhesion factors and trace elements, bouillons, peptones such as meat peptone, casein peptone, soya peptone, bactopeptone, and lactalbumin hydrolysate, extracts such as yeast extract, bovine hypophysene exrtact, chicken embryo extract or beef extract, buffer solutions and agarosic gels. These nanocells containing culture medium final products form a further subject-matter of the invention.

For manufacturing liquid and semi-solid culture medium final products the nanocells are worked into the aqueous part of the final product. The working-in of the nanocells is effected preferably through stirring at room temperature, wherein usual stirring apparatus are applied such as e.g. propeller stirring devices, slant-blade stirring devices or magnet stirring devices.

Instead of the nanocells also the corresponding nanocell-prephase may be worked into the aqueous part of the final formulation. The addition of the nanocell prephase into the aqueous part of the

final products is effected through stirring and preferably at a temperature < 50° C.

Solid culture medium final products such as e.g. powder media, granulates, tablets and lyophilisates are coated or loaded with nanocells by way of spraying or dipping. For certain solid culture medium final products it is advantageous to admix the nanocell in the dehydrated form to the solid substance mixture, wherein the dehydration of the nanocell generally is carried out by freeze drying or spray drying in the prescence of usual auxiliary agents such as e.g. maltodextrin.

The culture medium final products are preferably used for in vitro cultivation and examination of cells, fungi, bacteria, viruses, insects and plants, for in vitro or ex vivo cultivation and examination of tissue and organs, for freezing organs, tissue, cells, fungi, bacteria, viruses, insects and plants, for the transfer of organs, tissues and embryos, for therapy (adoptive immune therapy, cancer treatment), for the perfusion of organs such as e.g. kidney or liver, for the preservation of organs up to their transplantation, for zytogenetic, moleculargenetic, pharmacological, toxicological and metabolic examinations, for the uptake and transport or for the examination of functional and regulatoric mechanisms.

The invention is explained further by way of the subsequent examples.

Nanocell manufacturing examples

Example 1: soya lecithin 1.7% nanocell

Soya lecithin	1.70 %

Miglyol 812		3.45 %
Polysorbate 80		3.40 %
ethanol		1.40 %
10mM phosphate buffer, pH 6.0	ad	100.00 %

For manufacturing the nanocell Miglyol 812 and Polysorbate 80 are mixed into a homogeneous fluid phase. To this phase the lecithin dissolved in ethanol is added. From this there resulted a homogeneous solution which through stirring is added to the phosphate buffer. The obtained nanocell is sterile-filtered by way of a 0.2 µm filter.

Example 2: egg lecithin 1.4% nanocell

egg lecithin		1.40 %
Miglyol 812		2.85 %
Polysorbate 80		4.00 %
ethanol		1.40 %
10 mM phosphate buffer pH 6.0	ad	100.00 %

The manufacture of the nanocell is effected in a manner analogously to the Example 1.

Example 3: vitamin E 0.45% nanocell

vitamin E		0.45 %
soya lecithin		1.20 %
Miglyol 812		3.00 %
Polysorbate 80		2.90 %
ethanol		1.00 %
10mM phosphate buffer, pH 6.0	ad	100.00 %

For manufacturing the nanocell Polysorbate 80, Miglyol 812 and vitamin E are mixed into a homogeneous liquid phase. To this phase the lecithin dissolved in ethanol is added. From this there resulted a homogeneous solution which through stirring is added to the phosphate buffer. The obtained nanocell is sterile-filtered by way of a 0.2 µm filter.

Example 4: vitamin E acetate 2% nanocell

vitamin E acetate	2.00 %
soya lecithin	0.49 %
Miglyol 812	0.71 %
Polysorbate 80	1.86 %
ethanol	0.40 %
10mM phosphate buffer, pH 6.0	ad 100.00 %

The manufacture was effected in a manner analogous to Example 3.

Example 5 vitamin E acetate 1.2% nanocell

vitamin E acetate		1.20 %
Polysorbate 80		0.98 %
10mM phosphate buffer, pH 6.0	ad	100.00 %

For manufacturing the nanocell vitamin E acetate and Polysorbate 80 are mixed into a homogeneous liquid phase. The resulting clear solution is added to water through stirring. The obtained nanocell is subsequently sterile-filtered by way of a 0.2 µm filter.

Example 6: vitamin A palmitate 1.8% nanocell

vitamin A palmitate (1.7 Mio IU/g)	1.80 %
vitamine E acetate	0.10 %

soya lecithin		1.70 %
Miglyol 812		1.80 %
Polysorbate 80		3.15 %
ethanol		1.40 %
10mM phosphate buffer, pH 6.0	ad	100.00 %

The manufacture was effected in a manner analogous to Example 3.

Example 7: hydrocortisone 0.05% nanocell

hydrocortisone		0.05 %
soya lecithin		0.36 %
Miglyol 812		0.72 %
Polysorbate 80		0.71 %
ethanol		0.30 %
10 mM phosphate buffer, pH 6.0	ad	100.00 %

The manufacture was effected in a manner analogous to Example 3.

Example 8: omega-3 fatty acids 3.5% nanocell

Incromega E3322 (Croda)		5.50 %
vitamin E acetate		0.60 %
soya lecithin		0.30 %
Polysorbate 80		3.60 %
10mM phosphate buffer, pH 6	ad	100.00 %

For manufacturing the nanocell the omega-3 fatty acids (Incromega E3322, Croda) vitamin E acetate, Polysorbate 80 and soya lecithin are mixed into a homogeneous liquid phase. The resulting clear solution is added to the phosphate buffer through stirring. The obtained nanocell is subsequently sterile-filtered by way of a 0.2 µm filter.

Example 9: coenzyme Q10 0.5% nanocell

coenzyme Q10		0.50 %
soya lecithin		1.10 %
Miglyol 812		4.50 %
Polysorbate 80		3.40 %
propylene glycol		0.48 %
Ronoxan A (Roche)		0.02 %
10mM phosphate buffer, pH 6	ad	100.00 %

For manufacturing the nanocells Miglyol 812, polysorbate 80, propylene glycol, Ronoxan A and soya lecithin are mixed into a homogeneous liquid phase. To this mixture there is added the coenzyme Q10 through stirring and heating. From this there resulted a clear fluid which through stirring (e.g. a magnet stirring device) is added to the phosphate buffer which was heated previously to 50°C. The obtained nanocell was subsequently sterile-filtered by way of a 0.2 µm filter.

In the subsequent Table 1 the particle sizes of the nanocells are drawn up.

Table 1

nanocell	particle diameter[1] [nm]	standard deviation [nm]
soya lecithin 1.7% nanocell Example 1	15.6	5.9
egg lecithin 1.4% nanocell Example 2	15.5	5.2
vitamine E acetate 1.2% nanocell Example 3	19.1	6.9
vitamine E acetate 2% nanocell Example 4	22.2	9.9
vitamine E acetate 1.2% noncell Example 5	38.1	10.6
vitamine A palmitate nanocell Example 6	12.3	8.1
hydrocortisone 0.05% nanocell Example 7	15.1	6.3
omega-3 fatty acids 3.5% nanocell Example 8	56.6	21.2
coenzyme Q10 1% nanocell Example 9	22.1	9.0

[1] The particle diameter and the particle size distributions were determined by way of laser light scattering (Nicomp 370 Submicron Particle Sizer, Volume weighting)

As the subsequent table shows, nanocells also have an excellent storage stability:

Vitamine E acetate 2% nanocell (Example 4)

Table 2

storage condition		pH	particel diameter[2]	standard deviation	vitamine E acetate[3]
duration [months]	temp. [°C]		[nm]	[nm]	[%]
0		6.1	22.2	9.9	2.04
3	7	6.1	26.7	11.0	2.02
	25	6.1	28.4	11.3	2.04
	40	6.0	27.6	12.2	2.01
6	7	6.1	27.8	11.2	2.04
	25	6.0	29.1	11.8	2.03
	40	6.0	32.2	12.3	2.02
12	7	6.0	26.8	12.7	2.04
	25	6.0	29.1	13.6	2.03
	40	6.0	36.9	13.3	2.03

[2] The particle diameter and the particle size distributions were determined by way of laser light scattering (Nicomp 370 Submicron Particle Sizer, Volume weighting)
[3] The content of vitamin E acetate was determined by way of HPLC

Examples of culture medium final products containing nanocells.

Example 10: culture medium final product containing vitamin E acetate nanocells

Cellular uptake of vitamin E or vitamin E acetate

Microvascular endothelium cells of the cell line HMEC-1 were raised up to confluence, thereafter washed twice and incubated in a medium of the following composition:

vitamin E acetate nanocell according to example 4	0.0575 %
EGF (Laboserv, Staufenberg, Germany)	0.0050 %
hydrocortison (Sigma, Munich, Germany)	0.0001 %
foetal calf's serum (Biochrom, Berlin, Germany)	10.0000 %
MCDB-131 (Biochrom, Berlin, Germany)	ad 100.0000 %

After 96 hours the uptake of vitamin E by the cells was quantified. For this the cells were harvested with the help of trypsin/EDTA, washed, extracted with hexane and the concentration of vitamin E in the hexane extract was determined by way of HPLC. The pellets remaining after the hexane extraction were used for DNA examination. The results shown in Figure 1 show that vitamin E was efficiently taken up by the cells from cells out of the above medium. For comparison the cellular vitamin E concentrations of analogous trials are cited in which the same quantity of vitamin E or vitamin E acetate was added to the medium, not in the form of nanocells, but as a solution in ethanol or DMSO, as a solubilisate in foetal calf's serum or as a cyclodextrin complex.

Antioxidative capacity of vitamin E or vitamin E acetate

Microvascular endothelium cells of the cell line HMEC-1 were raised up to confluence, thereafter washed once and incubated for 96 hours in a medium of the following composition:

vitamin E acetate nanocell according to example 4		0.0575 %
EGF (Laboserv, Staufenberg, Germany)		0.0050 %
hydrocortison (Sigma, Munich, Germany)		0.0001 %
foetal calf's serum		10.0000 %
(Biochrom, Berlin, Germany)		
MCDB-131 (Biochrom, Berlin, Germany)	ad	100.0000 %

For determining the antioxidative protective effect of vitamin E or vitamin E acetate the free radicals generated in the natural metabolism were determined by way of a fluorometric method. For this purpose the cells preincubated in the above medium for 96 hours were charged with a non-fluorescing colourant (carboxy-H_2DCFDA/AM, molecular probes) which reacts intracellularly with the free radicals, in particular hydroperoxides and by way of this becomes a fluorochrome. The relative fluorescence resulting from this represents a measure for the quantity of released radicals. In the present experiment the relative flourescence over a time period of 150 minutes was measured. The results shown in Figure 2 show that the vitamin E acetate nanocells show a very good antioxidative effect with respect to intracellularly generated radicals. For comparison the relative fluorescence values of analogous trials are cited in which the same quantity of vitamin E or vitamin E acetate was added to the medium not in the form of nanocells, but as a solution in ethanol or DMSO, as a solubilisate in foetal calf's serum or as a cylcodextrin complex.

Zytotoxicity of vitamin E acetate nanocells

Microvascular endothelium cells of the cell line HMEC-1 were raised up to confluence, thereafter washed once and accommodated in a medium of the following composition:

EGF (Laboserv, Staufenberg, Germany)		0.0050 %
hydrocortisone (Sigma, Munich, Germany)		0.0001 %
foetal calf's serum (Biochrom, Berlin, Germany)		10.0000 %
MCDB-131 /Biochrom, Berlin, Germany)	ad	100.0000 %

The obtained cell suspensions were hereupon supplemented with 5, 10, 25, 50 and 75 µM vitamin E acetate, in the form of vitamin E acetate nanocells according to example 4 and incubated for 96 hours. The cytotoxic effect was determined with the help of the LDA assay. With this the release of the enzyme lactate dehydrogenase from dying cells in the supernatant was measured and represented as a percentage for control. The results shown in Figure 3 show that the vitamin E acetate nanocell, even in a high concentration is untoxic.

Stability of vitamin E acetate nanocells

Microvascular endothelium cells of the cell line HMEC-1 were raised up to confluence, thereafter washed once and accommodated in a medium of the following composition:

EGF (Laboserv, Staufenberg, Germany)	0.0050 %
hydrocortisone (Sigma, Munich, Germany)	0.0001 %
foetal calf's serum (Biochrom, Berlin, Germany)	10.0000 %

MCDB-131 (Biochrom, Berlin, Germany) ad 100.0000 %

The obtained cell suspensions were hereupon supplemented with 5, 10, 25, 50, 75 µM vitamin E acetate in the form of vitamin E acetate nanocells according to Example 4. At the point in time 0 and after 96 hours by way of HPLC the content of vitamin E acetate in the medium was determined. The decrease of the vitamin E actetate concentration fluctuated, according to the added concentration, between 7 and 29 %. This reduction is in contrast to other solutising agents extremely low.

Example 11: Culture medium final product containing vitamin A palmitate nanocells

Skin fibroblasts: cellular uptake of vitamin A palmitate

Human skin fibroblasts of the cell line HFP-1 were raised up to confluence, thereafter washed once and incubated in a medium of the following composition:

vitamin A palmitate nanocell according to Example 6		0.0095 %
1% penicillin/streptomycin		0.0050 %
foetal calf's serum (Biochrom, Berlin, Germany)		10.0000 %
DMEM (Dulbeccos's modified Eagle Medium, Biochrom, Berlin, Germany)	ad	100.0000 %

After 15 minutes, 2, 8 and 24 hours the uptake of vitamin A palmitate by the cells was qunatified. For this the cells were harvested at the cited points in time, washed, extracted with hexane and the concentration of vitamin A palmitate in the hexane extract was determined by way of HPLC. The pellets remaining after the hexane extraction were used for DNA

examination. The results shown in Figure 4 show that vitamin A palmitate is rapidly and efficiently taken up from the above medium into the cells. For comparison the cellular vitamin A palmitate concentration of an analogous trial is cited in which the same quantity of vitamin A palmitate was added to the medium not in the form of the nanocell but as a cyclodextrin complex.

Bronchial epithelium cells: cellular uptake of vitamin A palmitate

Human bronchial epithelium cells of the cell line NHBE were raised up to confluence, thereafter washed once and incubated in a medium of the following composition:

Vitamin A palmitate nanocell according to Example 6		0.0095 %
BEGM BulletKit	ad	100.0000 %
(Bronchial epithelial growth media, Clonetics)		

After 15 minutes, 2, 8 and 24 hours the uptake of vitamin A palmitate by the cells was qunatified. For this the cells were harvested at the cited points in time, washed, extracted with hexane and the concentration of vitamin A palmitate in the hexane extract was determined by way of HPLC. The pellets remaining after the hexane extraction were used for DNA examination. The results shown in Figure 5 show that vitamin A palmitate is rapidly and efficiently taken up from the above medium into the cells. For comparison the cellular vitamin A palmitate concentration of an analogous trial is cited in which the same quantity of vitamin A palmitate was added to the medium not in the form of the nanocell but as a cyclodextrin complex.

The Figures of the drawings mentioned in the previous examples 10 and 11 show in detail the following:

Figure 1: cellular uptake of vitamin E or vitamin E acetate from media, containing differing vitamin E or vitamin E acetate supplements. The quantities of vitamin E taken up by the cells (NHBE) are expessed in pmol/µg DNA. The cited values are average values of four independent trails.

Figure 2: antioxidative capacity of various vitamin E or vitamin E acetate supplements against intracellularly generated radicals. The cited values are average values +/- SD of four independent trails.

Figure 3: Zytotoxicity of vitamin E acetate nanocells with respect to HMEC-1 cells. The cited values are average values of four independent trials.

Figure 4: Cellular uptake of vitamin A palmitate out of media containing vitamin A palmitate nanocells or vitamin A palmitate cylcodextrin complexes. The quantities of vitamin A palmitate taken up by the skin fibroblasts (HFP-1) are expressed in µmol/µg DNA. The cited values are average values +/- SD of three independent trials.

Figure 5: Cellular uptake of vitamin A palmitate out of media containing vitamin A palmitate nanocells or vitamin A palmitate cylcodextrin complexes. The quantities of vitamin A palmitate taken up by the bronchial epithelium cells are expressed in µmol/µg DNA. The cited values are average values +/- SD of three independent trials.



Patent claims

1. The use of a nanocell containing as a preparative composition
   (a) a membrane-forming molecule
   (b) a co-emulsifier
   (c) a lipophilic component
   in culture medium final products.

2. The use according to claim 1, characterised in that the nanocell contains
   (a) as membrane-forming molecules, substances which are suitable for forming two-layer systems,
   (b) as co-emulsifiers, substances which preferably form O/W structures and
   (c) as a lipophilic component, a lipophilic substance usual for culture medium final products.

3. The use according to claim 1 or 2, characterised in that the nanocell as component
   (a) contains a natural or synthetic phospholipid, a hydrated or part-hydrated phospholipid or mixtures thereof.

4. The use according to one of the claims 1 to 3, characterised in that the component (a) is present in the nanocell used according to the invention in a concentration of 0.1 to 30% by weight with respect to the total weight of the components (a), (b) and (c).

5. The use according to one of the claims 1 to 4, characterised in that the nanocell as component (b) contains alkali metal-, ammonium- or amine salts of C_8-C_{20} fatty acids, saturated and unsaturated C_8-C_{18} alkyl sulphates, C_8-



C_{20} alkane sulphonates, fatty alcohol phosphoric acid esters, salts of the bile acid, invert soaps (quats), partial fatty acid esters of sorbitane, sugar esters of fatty acids, fatty acid partial glycerides, alkyl maltosides, alkyl glucosides, C_8-C_{18}-betaines, C_8-C_{18}-sulphobetaines or C_8-C_{24}-alkylamido-C_1-C_4-alkylene betaines, polyglycerine esters of fatty acids, polypropylene glycol esters of fatty acids, lactic acid esters of fatty acids, proteins, emulsifiers of the polyoxyethylene type or mixtures of these substances.

6. The use according to one of the claims 1 to 4, characterised in that the nanocell as component (b) contains emulsifiers of the polyoxyethylene type.

7. The use according to claim 6, characterised in that the nanocell as component (b) contains polyethoxylised sorbitane fatty acid esters, polyethoxylised vitamin E derivatives, polyoxyethylene-glycolised natural or hydrated vegetable oils, polyethoxylised fatty alcohols, polyethoxylised fatty acids, polyethoxylised fatty acid partial glycerides, polyethoxylised lanolin and its derivatives, polyethoxylised carbohydrates, blockcopolymerisates of ethylene oxide and propylene oxide, or mixtures of these substances.

8. The use according to one of the claims 1 to 7, characterised in that the component (b) is present in the nanocell used according to the invention in a concentration of 1 to 50 % by weight with respect to the total weight of the components (a), (b) and (c).

9. The use according to one of the claims 1 to 8, characterised in that the component (c) contains a natural, a



synthetic or a partial synthetic di- or triglyceride, a lipophilic substance usual for culture medium final products or mixtures of these substances.

10. The use according to claim 9, characterised in that the nanocell as component © contains a fat-soluble vitamin, a carotinoide, a ubiquinone, a flavonoid, an isoflavonoid, a polyphenole, a phytooestrogene, a lipophilic amino acid, a saturated or unsaturated fatty acid, an essential fatty acid, a lipid, a lipophilic antibiotic or antimyotic, a lipophilic hormone, a lipophilic marker for examining biological functions or a mixture of these substances.

11. The use according to one of the claims 1 to 10, characterised in that the component (c) is present in the nanocell applied according to the invention in a concentration of 0.1 to 70% by weight with respect to the total weight of the components (a), (b) and (c).

12. The use according to one of the claims 1 to 11, characterised in that the component (d) contains a C_2-C_8 alcohol.

13. The use according to one of the claims 1 to 12, characterised in that the culture medium final product is liquid, solid or semi-solid.

14. Liquid or semi-solid culture medium final products containing a nanocell as defined in claim 1.

15. Solid culture medium final product containing a nanocell as defined in claim 1.

16. A culture medium final product according to claim 14, characterised in that the nanocell is present in the aqueous phase of the culture medium final product.

17. A culture medium final product according to claim 15, characterised in that the nanocell is present in a dehydrated form.

18. A method for manufacturing a liquid or semi-solid culture medium final product according to claim 14, characterised in that the nanocells are worked into the aqueous part of the final product.

19. A method for manufacturing the solid culture medium final product according to claim 15, characterised in that the culture medium final products are coated or loaded with the nanocell.

20. A method for manufacturing the solid culture medium final product according to claim 15, characterised in that the nanocells are admixed in dehydrated form to the solid substance mixture.

21. The use of culture medium final products according to one of the claims 13 to 17 for in vitro cultivation and examination of cells, fungi, bacteria, viruses, bacteriophages, insects and plants, for in vitro or ex vivo cultivation and examination of tissue and organs, for freezing organs, tissue, cells, fungi, bacteria, viruses, bacteriophages, insects and plants, for the transfer of organs, tissues and embryos, for therapy (adoptive immune therapy, cancer treatment), for the perfusion of organs, for the preservation of organs up to their transplantation, for zytogenetic,

moleculargenetic, pharmacological, toxicological and metabolic examinations, for the uptake and transport studies or for the examination of functional and regulatoric mechanisms.

22. The use of a nanocell containing
(a) a membrane-forming molecule
(b) a co-emulsifier
(c) a lipophilic component
in culture medium final products, wherein the nanocell is obtainable by (α) mixing the components (a), (b) and (c) until there arises a homogeneous, clear fluid, called nanocell prephase, and (β) addition of the fluid obtained in step (α) into an aqueous part of the culture medium final products, characterised in that steps (α) and (β) are effected without further energy expense.