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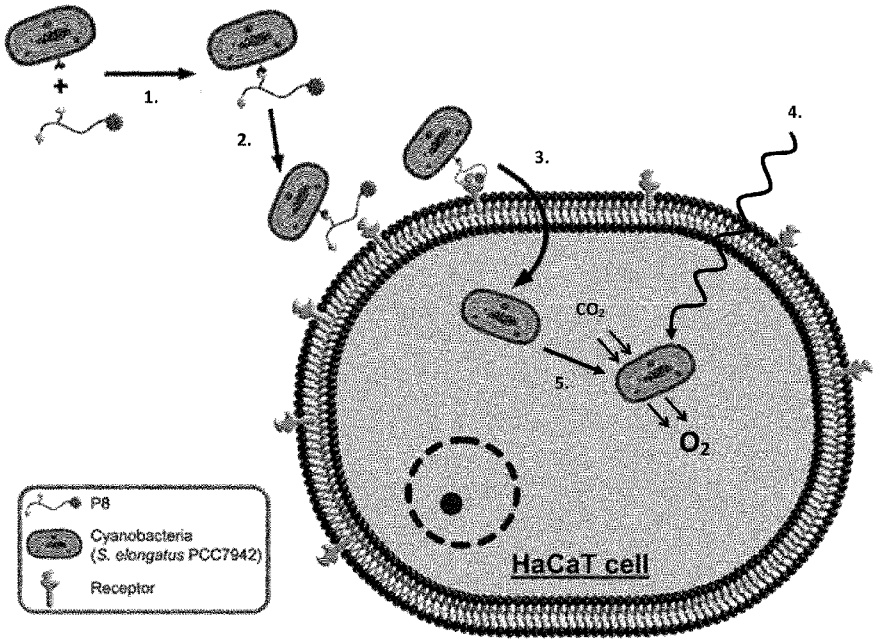

Fig. 1

(57) Abstract: Provided are novel methods for enhancing uptake of structures with a biological or equivalent membrane, or envelope or equivalent into host cells to provide these with a desired phenotypic characteristic, cells comprising the structures, compositions comprising the modified host cells and such cells and compositions for use in treatment of wounds or other diseases or for cosmetic application.

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Methods for enhanced cellular uptake of structures with a complex surface

FIELD OF THE INVENTION

The present invention generally relates to novel methods for enhancing uptake of structures with complex, respectively heterogenous surfaces, such as a biological or equivalent membrane or envelope into cells and provision of respectively altered cells and compositions comprising these and their use in medical treatment.

BACKGROUND OF THE INVENTION

Several methods are known for selectively targeting cells in a patient for delivery of molecules, such as various proteins, peptides, and nucleic acids, protein toxins, antisense oligonucleotides, liposomes or diagnostic or therapeutic agents, which include as classes the aforementioned molecules and in functional terms agents such as compositions for radiotherapy or for neutron capture therapy, compositions for chemotherapy, analgesics, antibiotics, antihypertensive agents, antiviral agents, antihistamines, expectorants, vitamins, plasmids, and the like. For example, folate conjugates have been used for the selective targeting of cell populations expressing folate receptors or other folate binding proteins to label or deliver bioactive compounds to such cells. Recently, a novel method has been described in WO 2019/057988 A1 which can be employed to trigger endocytosis and enables the target-specific uptake of molecules, proteins and other biofunctional material or particles into cells, when directly linked to them and which has particularly shown its applicability for uptake of nanoparticles with an uptake efficiency of about > 5 %. Concerning the introduction of more complex and in particular of living entities (cells) one publication appeared recently describing methods for the cellular uptake of cyanobacteria Synechococcus elongatus (*S. elongatus*) into eukaryotic cells: Agapakis et. al., PLoS One 6 (2011) e08877-8, used three different approaches, microinjection, cell invasion by genetically modified cyanobacteria and phagocytosis by macrophages in order to transfer cyanobacteria into different eukaryotic cells. From these methods', microinjection has shown the highest efficiency with about 5% as long as the host cell survives the relatively harsh procedure. However, this method is not applicable for large scale generation of endosymbiotic cells. Macrophages are well-known to efficiently phagocytose large numbers of bacteria. This cell type is part of the mammalian immune system and specialised to seek, engulf and digest foreign microparticles and bacteria. With a few exceptions, however, uptake

leads to destruction of cells. In addition, the cell invasion efficiency of genetically modified cyanobacteria into mammalian cells was below 5 % in all of these methods tested.

Cells generated by such methods could be used, in particular if the uptake rates could be increased and the introduced cell kept intact and in a functional state for a longer terms in the host cells, in several scenarios, *e.g.*, also therapeutic scenarios, where such cells altered by the introduction of a desired phenotype or compositions comprising these are used in treatment of humans or animals.

One of the many possible scenarios for the use of the cells generated by such methods is the treatment of non-healing wounds.

More than 5 million people suffer from non-healing wounds in Germany after severe burns or as a consequence of metabolic disorders caused by underlying diseases such as diabetes, arteriosclerosis or obesity. One central reason for the very poor healing of the skin tissue represents the drastic undersupply of oxygen (hypoxia) leading to more than 12.000 cases of limb amputations annually. Besides that, current therapies for the treatment of non-healing wounds include tissue transplantation or hyperbaric oxygen therapy. Chronic wound treatment leads to high annual health care costs of about 6 million EUR. For instance, the costs estimated for the treatment of a single diabetic ulcer are about 50,000 USD (Paquette, *et. al.* (2002)). Due to increased aging and obesity of the Western population, cases of patients suffering from non-healing wounds will further increase.

Wound healing is a complex physiological process that requires an ideal interplay among a number of highly regulated factors and molecular machineries, which have to function in concert for successful wound closure. Underlying metabolic defects caused by diseases such as diabetes or obesity can disturb the balance of important cascades in the wound healing process that can ultimately lead to chronic, nonhealing wounds. The current clinical gold standard for treating chronic wounds comprises wound debridement and excision of necrotic tissue, followed by skin transplantation for wound closure. In case of tissue hypoxia, the standard surgical treatment is ineffective and followed by wound healing failures and, ultimately, by amputations. Hyperbaric oxygen therapy has proven to be moderately efficient for hard to heal wounds but is solely available in specialised centres due to high costs. The patient is repeatedly exposed to a defined concentration of medically pure oxygen inside a

special pressure chamber for several hours. Systemic intake of relatively high oxygen concentrations via inhalation does not lead to the expected outcome of the procedure.

Alternative methods, including negative pressure wound therapy or vacuum-assisted wound closure are nowadays commonly used for temporary wound closure and induction of tissue granulation. These methods involve a wound dressing system which completely covers the wound and applies a negative pressure to the surface. Wound exudates are removed while maintaining a moist environment.

Another approach is to use cold plasma as a therapeutic agent to treat chronic wounds (medical application in 2015, ColdPlasmaTec). However, this technique is not specific to damaged or necrotic tissue and will not target hypoxia conditions that are underlying the chronic wound. Furthermore, only limited statistical data on success rates and potential long-term side effects associated with the method are currently available.

Accordingly, there is still requirement for improvement of the uptake efficiency in particular for more complex structures, in particular structures comprising or consisting of biological or equivalent membrane or envelope or their components such as cells and for effective and safe methods to enable and/or enhance the uptake of such structures into host cells a long-lasting or even permanent way, *i.e.* upholding the functionality of these structures after uptake in the host cells and preventing their degradation.

In addition, since current approaches for the treatment of chronic, non-healing wounds exhibit insufficient success rates, are highly expensive, or are still in its infancy and possibly associated with numerous side effects, it is necessary to develop new strategies.

Thus, the technical problem underlying the present invention was to provide methods for cellular uptake of complex structures, such as structures with a biological or equivalent membrane or envelope or equivalent into cells. The solution to the technical problem is achieved by providing the embodiments characterized in the claims and described further below.

BRIEF DESCRIPTION OF THE INVENTION

In a general aspect, the present invention relates to a method for providing a cell with a desired phenotypic characteristic, comprising:

a) incubating a structure provided with a biological or equivalent membrane or envelope and conjugated with a PAR binding ligand with a host cell expressing a protease activated receptor (PAR) on its surface under conditions allowing uptake of the structure into the host cell, wherein the structure or its content is capable of conferring the desired phenotypic characteristic to the host cell; and

b) detecting the cell with said phenotypic characteristic, and optionally isolating the cell.

Preferably, the phenotypic characteristic provided by this method is other than the presence or level of said structure or its content per se. The PAR binding ligand is preferably covalently attached to a linker molecule, which provides the ability to be chemically linked or associated to the components of a biological or equivalent membrane or envelope, wherein the linker comprises an aromatic moiety or system. Preferably the aromatic moiety is a fluorophore, such as 6-carboxyfluorescein (6-FAM) und 5-carboxyfluorescein (5-FAM), a mixture thereof or eosin, rhodamine or corresponding derivatives.

Preferably the structure provided with a biological or equivalent membrane or envelope used in the method described herein is a cell, further preferred wherein the cell is a prokaryotic cell, mostly preferred a cyanobacterium. As to the host cell, this is preferably a eukaryotic cell, more preferred a mammalian cell, even more preferred a human cell and most preferred a human keratinocyte.

The method described herein is preferably designed in that way that the desired phenotypic characteristic provided to the host cell is a gain of a phenotypic characteristic not present in the initial cell before the uptake of the structure or an increase or decrease of a phenotypic characteristic already present in the initial cell. Furthermore, preferably the desired phenotypic characteristic is an effect on production or consumption of a molecule, change of a response of the host cell to an added substance, effect on the ability of the host cell to grow or die (e.g., induction of death of a cell) in an environment or an observable alteration of a cultural, behavioral or morphological characteristic of the host cell.

In one embodiment the phenotypic characteristic provided by the method described herein is production of oxygen or reduced oxygen consumption by the host cell. The phenotypic characteristic can be also both, production of oxygen and reduced oxygen consumption by the host cell.

Provided herewith is also a cell obtainable by the method described herein. This cell is preferably a human keratinocyte which has been provided with the phenotypic characteristic of oxygen production or reduced oxygen consumption, compared with a cell not subjected to the method described herein, wherein preferably a cyanobacterium has been introduced into the cell.

The present application also relates to a composition or kit comprising the cell which has been provided with the phenotypic characteristic as described herein, wherein the composition is a pharmaceutic composition comprising a pharmaceutically acceptable carrier.

The cell or composition described herein is preferably designated for use in the treatment of wounds.

The application is finally also directed to a use of a PAR binding ligand for the generation of an endosymbiotic cell.

Brief description of the figures

Fig. 1 Schematic illustration of introduction of a prokaryotic cell into a eukaryotic cell by the method of the present invention. **1.** Conjugation of Cyanobacteria *Synechococcus elongatus* (PCC7942) with the novel molecular transporter molecule P8. **2.** P8-modified cyanobacteria bind to and activate target receptor molecules (protease activated receptor 2, PAR2) on the surface of cells, such as HaCaT cells. **3.** Binding to PAR2 mediates the cellular uptake (endocytosis) of cyanobacteria into the host cell. **4.** Exposure of the altered cell to light. **5.** Photosynthetic oxygen production by the Cyanobacterium within the neo-symbiontic keratinocyte.

Fig. 2 **A.** - Flow cytometry analysis of degree of P8-conjugation to the surface molecules of *S. elongatus* PCC 7942. Approximately 1 million cells of cyanobacteria PCC 7942 were incubated in 1 mL PBS containing 1 mM P8 molecules. The incubations were

carried out in the presence (blue curve) or absence (green curve) of the chemical coupling reagents EDC and NHS (red signal) for 1 h, in the dark at room temperature. After incubation, glycine (1 mM final concentration) was added to the reaction mix in order to inactivate remaining NHS ester. Samples were directly analysed in flow cytometer (green and blue curve) or further washed by subsequent centrifugation, followed by pellet-resuspension in PBS and afterwards analysed by flow cytometry (red and purple curve). Sample data were normalized on number of events and corresponding histograms were generated by plotting cyanobacterial cell count against the P8-fluorescence (λ_{ex} 488 nm, λ_{em} measured at 488 536 nm).

B, C. -Flow cytometry analysis of P8-attachment to the surface of cyanobacteria PCC 7942 after extensive washing. Approximately 1 million cells of cyanobacteria PCC 7942 were incubated in 1 mL PBS containing 1 mM P8 molecules. The incubations were carried in the presence (blue curve) or absence (red curve) of the coupling reagents EDC and NHS for 1 h, in the dark at room temperature. The samples were further treated with glycine (1 mM final concentration) and washed 5 times by subsequent centrifugation, followed by pellet-resuspension in PBS, and finally analysed by flow cytometry. Sample data were normalized on number of events. **B.** Dot plot for each sample was generated by plotting P8-fluorescence (λ_{ex} 488 nm, λ_{em} 536 nm) against fluorescence of cyanobacteria pigment phycocyanobilin (PCB, λ_{ex} 638 nm, λ_{em} 675 nm) and obtained plots were overlaid for evaluation. **C.** Corresponding histogram (stacked mode) of **B** illustrating cyanobacterial cell count plotted against P8-fluorescence (λ_{ex} 488 nm, λ_{em} 536 nm). Curves – from top to bottom: green, red, blue, purple, yellow/orange.

D, E - Flow cytometry analysis of time dependent dissociation of P8 from the surface of cyanobacteria PCC 7942. Approximately 1 million cells of PCC 7942 were incubated in 1 mL PBS containing 1 mM P8 molecules in the presence of coupling reagents EDC and NHS for 1 h, in the dark at room temperature. After incubation samples were treated with glycine (1 mM final concentration) and subsequently centrifuged, followed by pellet-resuspension in PBS. Cyanobacteria were further incubated in DMEM without HaCaT cells for 15 h, in the dark at 30°C and samples were analysed by flow cytometry. Sample data were normalized on number of events. **D.** Dot plot for each sample was generated by plotting P8-fluorescence (λ_{ex} 488 nm, λ_{em} 536 nm) against fluorescence of cyanobacteria pigment phycocyanobilin (PCB, λ_{ex} 638 nm, λ_{em} 675 nm) and obtained plots were overlaid for evaluation. **E.**

Corresponding histogram (overlay mode) of **D.** illustrating cyanobacterial cell count plotted against P8-fluorescence (λ_{ex} 488 nm, λ_{em} 536 nm)

Fig. 3 P8-mediated uptake of *S. elongatus* into HaCaT cells. Epifluorescence microscopy images of HaCaT cells incubated with cyanobacteria PCC7942 under different conditions are illustrated. Untreated cyanobacteria (A1-C1: **PCC7942**), cyanobacteria incubated only in the presence of soluble P8 (A2-C2: **PCC7942 + P8**) or cyanobacteria covalently conjugated with P8 (A3-C3: **PCC7942(P8)**) were incubated with HaCaT cells for 15 h under culturing conditions, respectively. A clear enhanced uptake can be seen for cyanobacteria covalently conjugated with P8 (A3-C3) relative to the other two samples (A1-C1, A2-C2). A first statistical analysis of the uptake efficiency based on this figure indicated a much higher efficiency than reported in the prior art (5%), namely an efficiency in the approximate range of about 40%.

Fig. 4 P8-mediated uptake of PCC7942 into HaCaT cells. Confocal laser scanning microscopy images of HaCaT cells showing endocytosed cyanobacteria PCC7942 conjugated with P8.

Fig. 5 Immunocytochemical (ICC) staining of cultured primary keratinocytes isolated from human skin tissue samples. 90,000 cells/ cm^2 of primary keratinocytes were seeded on each coverslip and incubated in 300 µL KGM2 medium without antibiotics for 24 h at 37°C, 5% CO_2. Keratinocyte specific intermediate filaments were detected using primary antibody directed against CK-14 (1:500 dilution in PBS containing 3% goat serum) and corresponding FITC-conjugated secondary antibody. Another primary antibody specifically binding the intracellular C-terminus of PAR2 was used in combination with Cy3-conjugated secondary antibody to image the localisation of the receptor (1:200 dilution in PBS containing 3% goat serum). **A-D.** Nuclei, PAR2 and CK-14 staining of isolated primary keratinocytes was performed using corresponding secondary antibodies. **E-H.** Negative control to indicate potential background signals caused by secondary antibody was performed without the addition of primary antibody to the primary keratinocytes. Samples were analysed by epi-fluorescence microscopy. Scale bars: 50 µm.

Fig. 6 Flow cytometry analysis demonstrating HaCaT cell lysis. Approximately 3 million HaCaT cells were resuspended in 100 µL PBS and mechanically lysed as described under Methods (section 3.4). Cell lysis was characterised using flow cytometry. HaCaT cytosol extract was separated from large size cell debris by centrifugation of entire HaCaT cell lysate at 14,000 rpm for 10 minutes at room temperature. Dot plots

were generated by plotting forward scatter (FSC) against side scatter (SSC). HaCaT cell populations are marked with the green gate 'Viable HaCaT'. **A.** PBS control. **B.** HaCaT cells before lysis **C.** Mechanically lysed HaCaT cells showing a clear reduction in cell count within the gated region relative to B.

Fig. 7 Time dependent cell viability of cyanobacteria PCC 7942 incubated in cytosol extracts isolated from HaCaT cells. Samples were analysed by flow cytometry. Approximately 1 million cyanobacteria PCC 7942 were inoculated in cytosol extracts of approximately equal number of HaCaT cells for up to 48 hours in a phytotron at 30°C with 12 hours light and dark phases. After **A.** 3, **B.** 24 and **C.** 48 hours of incubation either in cytosol extracts (black curves) or 30% ESAW medium (red curves), fluorescence of cyanobacteria pigment phycocyanobilin (PCB) was measured as an indicator for cell viability. Histograms were generated by plotting cell count of cyanobacteria PCC 7942 against PCB fluorescence (λ_{ex} 638 nm, λ_{em} 675 nm). All curves were normalized on the number of events measured in 30 µL sample volume.

Fig. 8 Vitality of cyanobacteria PCC 7942 in the presence of coupling reagents NHS and EDC analysed by flow cytometry. Approximately 1 million cells of cyanobacteria PCC 7942 were incubated with coupling reagents EDC and NHS at a final concentration of 10 µM and 20 µM respectively. Reaction mixtures were incubated on a rotating wheel, in the dark at room temperature for 1 hour. Dot plot for each sample was generated by plotting fluorescence of cyanobacteria pigment phycocyanobilin (PCB, λ_{ex} 638 nm, λ_{em} 675 nm) against fluorescence of allophycocyanin (APC, λ_{ex} 638 nm, λ_{em} 748 nm). The PCB-positive cyanobacteria population was marked with the red gate 'PCC 7942' **A.** PBS control. **B.** Cyanobacteria PCC 7942 before addition of coupling reagents NHS and EDC. **C.** Cyanobacteria PCC 7942 after addition of coupling reagents NHS and EDC.

Fig. 9 **A-B.** Potential intercellular crosslinking of cyanobacteria PCC 7942 in the presence of EDC and NHS. Crosslinking potential was analysed using flow cytometry. Approximately 1 million cells of cyanobacteria PCC 7942 were incubated with 4 µM or 20 µM EDC and NHS at their final concentrations in 1 mL PBS. The reaction mixture was incubated on a rotation wheel, in the dark, at room temperature for 1 hour. Incubations were stopped or 1 mM glycine was added and incubations were resumed under same conditions for 15 minutes. A dot plot for each sample was generated by plotting fluorescence of cyanobacteria pigment phycocyanobilin (PCB, λ_{ex} 638 nm, λ_{em} 675 nm) against forward scatter (FSC) and obtained plots were

overlaid for evaluation. Incubations were centrifuged at 5000 rpm, for 10 minutes at room temperature and cyanobacterial pellets were resuspended in PBS. **A.** Cyanobacteria PCC 7942 incubated with 20 µM EDC and 20 µM NHS **B.** Cyanobacteria PCC 7942 incubated with 4 µM EDC and 4 µM NHS. **C.** Photometric analysis of relative fluorescence intensity of Fmoc-Lys(5-Fam). Excitation wavelength of 485 nm and an emission wavelength of 535 nm was used and obtained relative fluorescence values were plotted against various concentrations of Fmoc-Lys(5-Fam) in mM.

Fig. 10 Oxygen production of cyanobacteria determined with the microfluidic-based oxygen sensing platform. The relative oxygen concentration in the media of cultured cyanobacteria (*Synechococcus sp.*) at 30 °C was measured over time (hours). Five independent sensor spots each providing a separate read out (indicated by coloured lines) are integrated into the microfluidic device. Measurements were taken every 10 min. Cyanobacteria were illuminated for 12 h (yellow bar) followed by an interval of 12 h in the dark (black bar). Two light/dark-cycles were performed in total.

Fig. 11 Growth curves of PCC7942 under selected culturing conditions. Cyanobacteria PCC7942 (sweet water strain) were cultivated at 30°C and 30% salinity (relative to ESAW (Enriched seawater, artificial water medium (Berges *et al.* 2001)) with different pH for 17 days. Cells were counted by flow-cytometry. Applied culturing conditions comprise a temperature of 30°C, a pH of 7.42 and a salt concentration of about 30% of that of ESAW mimicking three essential factors of cytosolic conditions within human keratinocytes.

Fig. 12 Biocompatibility of HaCaT cells co-cultured with *Synechococcus sp.* **A:** 150,000 HaCaT cells were seeded, grown for 72 h and afterwards co-cultivated with 0 (control), 300,000, 600,000 or 1,200,000 viable bacteria of *Synechococcus* sp. For 72 h. The number of viable HaCaT cells did not differ significantly between different treatments ($p = 0.485$). **B:** HaCaT cells were seeded as described above but incubated with cell lysate of 0 (control), 300,000, 600,000 or 1,200,000 *Synechococcus* sp. For 72 h. The number of viable HaCaT cells did not differ between different treatments ($p = 0.299$).

Fig. 13 Chemical conjugation of PAR2 agonist P8 molecules to surface molecules of cyanobacteria PCC 7942 analysed by flow cytometry. Approximately 1 million cells of cyanobacteria PCC 7942 were incubated in 1mL PBS containing 10 µM EDC, 20 µM NHS and 1 mM P8. The reaction mixture was incubated on a rotation wheel, in

the dark, at room temperature for 1 hour. After 1 hour of incubation, glycine was added (1 mM final concentration) and incubations were resumed under same conditions for 15 minutes. Incubations were centrifuged at 5000 rpm, for 10 minutes at room temperature and cyanobacterial pellets were resuspended in PBS. Dot plot (contour type) for each sample was generated by plotting P8-fluorescence (λ_{ex} 488 nm, λ_{em} 536 nm) against fluorescence of cyanobacteria pigment phycocyanobilin (PCB, λ_{ex} 638 nm, λ_{em} 675 nm). Gated regions (purple squares) indicate viable cyanobacterial populations **A.** Incubation of cyanobacteria PCC 7942 with P8 in the presence of EDC and NHS. **B.** incubation of only cyanobacteria PCC 7942. **C.** incubation of PBS.

Fig. 14 Time dependent interaction study of P8-conjugated cyanobacteria with HaCaT cells. Approximately 1 million P8-conjugated cyanobacteria resuspended in PBS were added to 90,000/cm^2 HaCaT cells in a 24 well plate. A ratio of about 10 cyanobacteria per HaCaT cell was maintained. The plate was incubated for up to 15 hours at 30°C in the dark. Incubations were stopped at time points of 3, 6, 9 and 15 hours by washing, performing cell fixation followed by cellular permeabilization as described under Methods. Cytokeratin was stained using anti-CK-14 specific primary antibody (1:500 dilution in PBS containing 3% goat serum) and corresponding Cy3-conjugated secondary antibody whereas cell nuclei were stained using a ready to use DAPI containing mounting solution (VectaShield, Vector Labs). **A-D, A1-D1, A2-D2.** Images illustrating a merge of nuclei (DAPI, blue) and cyanobacteria (PCB, red) staining, whereas the monochromatic images (**Aa-Da, A1a-D1a, A2a-D2a**) show only the PCB staining. **A-D**. HaCaT cell incubated without cyanobacteria. **A1-D1**. HaCaT cells incubated with unmodified cyanobacteria. **A2-D2**. HaCaT cells incubated with P8-conjugated cyanobacteria. All images were captured using epifluorescence microscope. Scale bar: 50 µm.

Fig. 15 Interaction study of P8-conjugated cyanobacteria PCC 7942 with cultured HaCaT cells analysed by epifluorescence microscopy. Approximately 1 million cyanobacteria PCC 7942 having different chemical modifications as described under Methods were added to 90,000/cm^2 HaCaT cells in a 24 well plate. A ratio of about 10 cyanobacteria PCC 7942 per HaCaT cell was maintained. The plate was incubated for 15 hours at 30°C in the dark using varying incubation conditions detailed described under Methods. Incubations were stopped after 15 hours by washing and subsequent cell fixation followed by cellular permeabilization as described under Methods. Nuclei

were stained using ready to use DAPI mounting solution (VecaShield). **A-H.** Images were captured using epifluorescence microscope. **A.** HaCaT cells incubated with P8-conjugated cyanobacteria PCC 7942. **B.** HaCaT cells incubated with cyanobacteria PCC 7942, which were previously incubated with soluble P8 in the absence of coupling reagents EDC and NHS. **C.** HaCaT cells incubated with soluble P8. **D.** HaCaT cells only. **E.** HaCaT cells incubated with NHS-modified cyanobacteria PCC 7942, which were not glycine treated post chemical conjugation reaction. **F.** HaCaT cells incubated with NHS-modified cyanobacteria PCC 7942, which were glycine treated post chemical conjugation reaction. **G.** HaCaT cells incubated with unmodified cyanobacteria PCC 7942 in the presence of soluble P8. **H.** HaCaT cells incubated with unmodified cyanobacteria PCC 7942. Scale bar, 50 µm. Monochromatic images (A1-H1) show again only cyanobacteria (PCB, red).

Fig. 16 Extensive washing of P8-conjugated cyanobacteria PCC 7942 preparations and consequential effects on interactions with cultured HaCaT cells. Approximately 1 million cyanobacteria PCC 7942 having different chemical modifications were washed 5 times prior to incubation with cultured HaCaT cells as described under Methods. Washed cyanobacteria PCC 7942 were subsequently added to 90,000/cm^2 HaCaT cells in a 24 well plate. A ratio of about 10 cyanobacteria PCC 7942 per HaCaT cell was maintained. The plate was incubated for 15 hours at 30°C in the dark using varying incubation conditions detailed described under Methods. Incubations were stopped after 15 hours by washing and subsequent cell fixation followed by cellular permeabilization as described under Methods. Nuclei were stained using ready to use DAPI mounting solution (VecaShield). **A-D.** Images were captured using epifluorescence microscope. **A.** HaCaT cells incubated with P8-conjugated cyanobacteria PCC 7942. **B.** HaCaT cells incubated with cyanobacteria PCC 7942, which were previously incubated with soluble P8 in the absence of coupling reagents EDC and NHS. **C.** HaCaT cells only. **D.** HaCaT cells incubated with unmodified cyanobacteria PCC 7942. Scale bar, 50 µm. **A1-D1** -PCB staining of cyanobacteria.

Fig. 17 Cellular localisation of P8-conjugated cyanobacteria PCC 7942 after 15 h of incubation with cultured HaCaT cells using confocal laser scanning microscopy (CLSM). Approximately 1 million P8-conjugated cyanobacteria PCC 7942 resuspended in PBS were added to 90,000/cm^2 HaCaT cells in 500 µL DMEM in each well of a 24 well plate. A ratio of about 10 cyanobacteria PCC 7942 per HaCaT cell was maintained. The plate was incubated for 15 hours at 30°C in the dark. Incubations

were stopped after 15 hours by washing and subsequent cell fixation followed by cellular permeabilization and immunocytochemical staining as described under Methods. Cytokeratin was stained using anti-CK-14 specific primary antibody (1:500 dilution in PBS containing 3% goat serum) and corresponding Cy3-conjugated secondary antibody whereas cell nuclei were stained using a ready to use DAPI containing mounting solution (VectaShield, Vector Labs). **A-D.** Overlay of the channels (Nuclei, CK-14 and cyanobacteria PCC 7942) showing colocalised fluorescence signals of cyanobacteria PCC 7942 (red signals) and HaCaT cells. Images were captured by confocal laser scanning microscopy (CLSM). Scale bar: **A.** 50 µm. and **B-D.** 20 µm.

Fig. 18 Z-axis scanning of HaCaT cells incubated with P8-conjugated cyanobacteria PCC 7942 using confocal laser scanning microscopy. Z-axis scan C' and D' was performed on samples C and D shown in Fig. 17. Images indicate localisation of cyanobacteria PCC 7942 (red signals) within the cytosol of cultured HaCaT cells. **C'.** Z-axis scan over a total layer thickness of 6 µm with 4 slices of 2 µm thickness respectively**. D'.** Z-axis scan over total layer thickness of 6.9 µm with 4 slices of 2.3 µm thickness respectively. Scale bar: 20 µm.

Fig. 19 Experimental control conditions supporting the localisation of P8-conjugated cyanobacteria PCC 7942 within the cytosol of cultured HaCaT cells. Approximately 1 million cyanobacteria PCC 7942 having different chemical modifications as described under Methods were added to 90,000/cm^2 HaCaT cells in 500 µL DMEM in each well of a 24 well plate. A ratio of about 10 cyanobacteria PCC 7942 per HaCaT cell was maintained. The plate was incubated for 15 hours at 30°C in the dark. Incubations were stopped after 15 hours by washing and subsequent cell fixation followed by cellular permeabilization and immunocytochemical staining as described under Methods. Cytokeratin was stained using anti-CK-14 specific primary antibody (1:500 dilution in PBS containing 3% goat serum) and corresponding Cy3-conjugated secondary antibody whereas cell nuclei were stained using a ready to use DAPI containing mounting solution (VectaShield, Vector Labs). Images were captured by confocal laser scanning microscopy (CLSM). **A-G** (first column). Overlay of the channels (Nuclei, CK-14 and cyanobacteria PCC 7942). Samples **A-F.** were incubated with primary anti-CK-14 and corresponding Cy3-conjugated secondary antibody **A.** HaCaT cells incubated with cyanobacteria PCC 7942, which were previously incubated with soluble P8 in the absence of coupling reagents EDC and NHS. **B.**

HaCaT cells incubated with NHS-modified cyanobacteria PCC 7942, which were not glycine treated post chemical conjugation reaction. **C.** HaCaT cells incubated with NHS-modified cyanobacteria PCC 7942, which were glycine treated post chemical conjugation reaction. **D.** HaCaT cells incubated with unmodified cyanobacteria PCC 7942. **E.** HaCaT cells only. **F.** HaCaT cells incubated in the presence of soluble P8. **G.** HaCaT cells incubated in absence of primary and secondary antibodies. Scale bar: 50 µm.

DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to a method for providing a cell with a desired phenotypic characteristic. Preferably this method comprises the steps of:

a) incubating a structure provided with a biological or equivalent membrane, or envelope and associated with a PAR binding ligand with a host cell expressing a protease activated receptor (PAR) on its surface under conditions allowing uptake of the structure into the host cell, wherein the structure or its content is capable to confer the desired phenotypic characteristic to the host cell; and

b) detecting the cell altered in said phenotypic characteristic, *i.e.* having said phenotypic characteristic.

Optionally, the cell is recovered or isolated after step b). Whether or not the cell has obtained the desired phenotypic characteristic can be determined by comparison with a wild type or initial cell, *i.e.* a host cell which has not been subjected to the method. In one preferred embodiment, the cell is provided with the desired phenotypic characteristic for a longer term, preferably for more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 hours.

Protease-activated receptors (PAR), their ligands and methods for generating new synthetic PAR, in particular PAR2- agonists are described in the international patent application WO 2019/057988 A1 by the present inventors, with the exemplary new PAR2-agonist P8 shown in Fig. 1, its synthesis shown in Fig. 2 and described in Examples 1 and 2 on pages 12-17 of the WO 2019/057988 A1, the content of which is incorporated herein by reference in its entirety.

In brief, Protease-activated receptors (PAR) are G-protein coupled seven transmembrane domain receptors (GPCR), located on the cell surface of a variety of cell types including keratinocytes, several immune cells such as neutrophils, monocytes (and other immune cells), pancreatic beta-cells and dendritic cells (Rattenholl & Steinhoff, 2008). Since PAR are coupled to intracellular guanyl nucleotide binding proteins (G-proteins), they induce signal transduction in response to a variety of stimuli from exterior to the interior of a cell. They in turn play an important role in cell survival, differentiation, inflammation and many other important cellular processes (Sharlow, *et. al.*, 2000). The PAR family consist of 4 different members in humans termed PAR1, PAR2, PAR3 and PAR4 (Soh, *et. al.*, 2010). In contrast to the classical ligand-induced GPCR activation, PAR activation occurs when certain proteases hydrolyse the extracellular amino terminus of receptor creating a tethered ligand (TL) that stimulates intracellular signal transduction (Cottrell, *et. al.*, 2003). The TL binding leads to activation of G-proteins and subsequent stimulation of various downstream processes.

PAR2 is activated by proteolytic action of trypsin, tryptase, and several coagulation factors. Proteolysis is an irreversible process, hence permanent cell stimulation by the TL is prevented *via* β-arrestin-mediated and clathrin-dynamin dependent endocytosis of receptor itself (Ricks & Trejo, 2009). Apart from natural protease activation, PAR2 was also shown to be activated by synthetic hexapeptides mimicking the exposed TL after proteolytic digest. The synthetic PAR2 agonist peptides, Ser-Leu-Ile-Gly-Lys-Val (SLIGKV-OH), Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL-OH) (Nystedt, *et. al.*, 1995) and their amidated forms Ser-Leu-Ile-Gly-Lys-Val-amide (SLIGKV-NH_2) Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL-NH_2) (Bohm, *et. al.*, 1996; Hollenberg, *et. al.*, 1996) mimic the tethered ligand of PAR2. These synthetic peptides were demonstrated to be able to activate PAR2. Along this line, a novel synthetic PAR2-agonist ligand molecule termed P8 (WO 2019/057988 A1) was used in the experiments and methods described herein.

The P8 molecule basically consists of 4 individual building blocks: hexapeptide SLIGRL-NH_2 activates PAR2. A diethylene glycol-moiety as a spacer unit provides a defined distance between the hexapeptide and a lysine residue. Lysine linker molecule has an amine group which can be utilized for chemical conjugation with suitable cargos. A fluorophore 5-carboxyfluorescein (5-FAM) is conjugated to the lysine and enables the photochemical detection and quantification of P8 molecule. This aromatic moiety has also been demonstrated to drastically increase the affinity of the entire P8 molecule to PAR2 (WO 2019/057988 A1).

The functionality of P8 molecule was demonstrated by analysing downstream effects as a consequence of PAR2 activation and signal transduction in PAR2 expressing cells using flow cytometry. In addition, enhanced endocytosis of P8-conjugted nanoparticles (NPs) into PAR2-expressing HaCaT cells relative to bare NPs has been demonstrated previously (WO 2019/057988 A1), however, as mentioned before rather on homogeneous, simple structures.

The method of the present invention is based thereon that a PAR binding ligand is associated to the surface of the structure to be taken up by the host cell. This association can be achieved in a non-covalent way, *e.g.*, by binding the PAR binding ligand to a phospholipid and by introducing this phospholipid into the surface of the structure. Preferably the association is obtained by a chemical reaction covalently binding (conjugating) the PAR binding ligand with components of the surface of the structure. Accordingly, in a preferred embodiment, the structure provided with a biological or equivalent membrane, or envelope is conjugated with a PAR binding ligand.

To avoid potential clumping of the structures due to the conjugation process with the PAR ligands, preferably a neutral amino acid, such as glycine is optionally added after the structure has been incubated with the PAR binding ligand (incubation reaction) and after incubation with the neutral amino acid the structures are pelleted by centrifugation, separated from the medium used in the incubation reaction and resuspended in a medium suitable for the incubation with and uptake with the host cells. Preferably, the conjugation reaction is performed for a time of 5, 10, 15, 20, 25, 30, 40, 45, 50, 60 minutes, or for several hours such as 1,5 hours, 2, 3, 4, 5 hours or even longer, overnight, or for 8, 10, 12, 14, 16 hours. The subsequent additional incubation with the neutral amino acid is preferably performed for a time between 1 and 60 minutes, preferably for 5, 10, 15, 20, 25, 30, 40, 45, 50 or 60 minutes. If living structures, such as cells are used, the incubation with the neutral amino acid is not performed for longer than for 15 minutes to avoid adverse effects on the vitality of the living structures. After the initial incubation of the conjugation reaction, or if performed, the additional incubation, the structures are preferably pelleted by centrifugation and resuspended in a suitable medium or solvent for the cellular uptake into the host cells. Structures conjugated with the PAR binding ligand are separated from non-conjugated structures before the incubation for the uptake with the host cells, however, in view of the fact, that non-conjugated structures are barely taken up by the host cells, this step is merely optional.

Preferably the desired phenotypic characteristic provided to the host cell by the method of the present invention is other than the presence or level of said structure or its content per se.

Whether or not the cell has obtained the desired phenotypic characteristic is preferably determined according to the method of the present invention by comparison with a host cell which has not been incubated with the structure (initial cell).

The phenotypic characteristic provided to the cell may be apparent directly after the introduction of the structure into the host cell or an additional induction may be required due to which the phenotypic characteristic is provided. The induction may occur via addition or administration of a substance or a compound, change of the components of a culture medium in which the host cells are or via a physical stimulus, such as a temperature change or irradiation, *e.g.*, with light.

The PAR binding ligand may be directly bound to the components of the biological or equivalent membrane, or envelope or equivalent of the structure. Preferably, however, a molecule used as an anchor or linker (molecule) is located between the components of the biological or equivalent membrane, prokaryotic cell wall or equivalent or envelope of the structure and the PAR binding ligand.

Preferably, the PAR binding ligand is covalently attached to a linker molecule which provides the ability to be chemically linked or associated to the components of a biological or equivalent membrane, or envelope or equivalent. Accordingly, the linker has a role which can be described as an anchor or attachment unit/molecule for the above-discussed connection of the PAR binding ligand with the surface components of the structure to be taken up into the cell to be modified by the herein described method (host cell).

In a preferred embodiment of the method described therein the linker molecule is lysine.

The linker can be attached via different means to the ligand, either directly or indirectly. In a preferred embodiment the linker molecule is attached to the ligand by a spacer molecule. Different molecules are suitable to be used as a spacer molecule. In a further preferred embodiment of the conjugate of the present invention the linker is attached to the ligand by a spacer molecule. Like linkers, spacer molecules such as those consisting of ethylenglycol units

are well known in the field; see, *e.g.*, for review Hamley, Biomacromolecules 15 (2014), 1543−1559 as well as Chen (2013) and Accardo (2014), *supra*. Preferably, the spacer molecule is diethylenglycol (DEG). However, it should be noted, that based on the molecular modelling data provided in Example 3 the spacer can be modified in length and structure to modulate the interactions between PAR and the ligand. In particular, this includes strengthening the binding by interactions outside of the binding site for the activating peptide. For example, while two ethylenglycol units (DEG) have been used in the Examples, it may well be extended to oligo- and polyethylenglycol (PEG) depending on specific demands of an application. For example, when the PAR-ligand, *e.g.* PAR2 activating peptide will be attached to complex respectively heterogeneous surfaces of micro vesicles or other nano-/micro-particles, it might be critical that the PAR2 activator peptide is more exposed to the environment and distant from the carrier surface. Accordingly, in a preferred embodiment of the method described herein, diethylenglycol (DEG) or polyethylenglycol (PEG) are used forming a PAR binding ligand-spacer-linker molecule.

Furthermore, in the method described herein the PAR binding ligand is preferably a PAR activating peptide or derivative thereof. To simplify the binding of the linker to the PAR binding ligand, preferably the peptide has a serine at the N-terminus and/or the carboxylate at the C-terminus modified to a primary amide, wherein preferably the linker is directly or indirectly attached to the N-terminus of the peptide at the free amine of the serine, however, mutation of the serine to threonine retained ligand activity, suggesting that the correct positioning of a polar group, such as sulphydryl or hydroxyl group of the amino acid side chain, as in serine or threonine is important for ligand binding. Therefore, other amino acids such as threonine or functional groups may be present at the N-terminus of the PAR ligand for attaching the linker and spacer, respectively.

In a preferred embodiment of the method described herein above and below, the PAR activating peptide comprises the amino acid sequence SLIGRL (SEQ ID NO: 1), SLIGKV (SEQ ID NO: 2). Alternatively, a derivative of these peptides is used, preferably SLIGRL-NH_2 or SLIGKV-NH_2. Preferably the PAR binding ligand is the PAR2 activating peptide SLIGRL which is preferably N-terminally conjugated to a spacer and linker molecule, in cases where the linker molecule is used as well.

The inventors initially envisioned to add a "detectable label" to facilitate detection of the PAR activating peptide and, after the uptake of the structure into a host cell also the detection of the structure within the host cell. However, during preliminary experiments the inventors observed that the aromatic moiety/system of the fluorophore significantly increased the affinity of P8 to PAR2 while remaining its activation and triggering endocytosis potential. So preferably at this position an aromatic moiety is provided at the linker or as part of the linker providing the ability to add additional interaction potential of the PAR activating ligand, in particular of P8 to a PAR, wherein the PAR is preferably PAR2. Accordingly, in a preferred embodiment of the method of the present invention the linker comprises a molecule with an aromatic moiety/system or aromatic functional group respectively. If it is intended not only to increase the interaction between the PAR activating ligand and the PAR but also to allow an easy detection of the PAR binding ligand and the structures with which it is associated or coupled to, the aromatic moiety as part of the linker is a detectable label, preferably a fluorophore. Accordingly, in a preferred embodiment of the method described herein the linker comprises a detectable label, preferably a fluorophore such as 6-carboxyfluorescein (6-FAM) and 5-carboxyfluorescein (5-FAM) or a mixture thereof or eosin, rhodamine or corresponding derivatives.

As indicated above, PARs of different organism can be used, depending also in particular on the fact, to which species the host cells belong, which are intended to be modified with the method described herein. In a preferred embodiment the PAR is PAR2, preferably human PAR and human PAR2, respectively.

The structure to be introduced into the host cells can be artificial or completely or partially natural as long an association or coupling with a PAR binding ligand is possible. Accordingly, the surface of the structure can comprise or consist of lipids, in particular lipids as present in biological membranes, such as phospholipids or can comprise or consist of proteins, peptidoglycans, glucuronic and galacturonic acid, acidic sugars or in principle all glycosaminoglycans (GAGs), glycoproteins, glycoprotein S-layers, pseudopeptidoglycan, or polysaccharides or mixtures of any of the aforementioned, *i.e.* comprise two, three, four, five, six or more of the aforementioned component types. In a preferred embodiment however, the structure provided with a biological or equivalent (such as synthetic membranes and envelopes) membrane, or envelope or equivalent is selected from the group consisting of: (micro)vesicles, nanoparticles (respectively liposomes or micelles), extracellular vesicles, endo- and exosomes,

viruses, virus-like particles, mitochondria, other cellular organelles or cells. In a particularly preferred embodiment, the structure is a cell.

According to the method described herein, if the structure is a cell, this cell can be either eukaryotic or prokaryotic. However, in a preferred embodiment, the cell to be introduced into the host cell with the method of the present invention is a prokaryotic cell.

In accordance with the preliminary results obtained in experiments performed in the context of the present invention (see Example 4 and Fig. 3), the method of the present invention leads to much higher uptake efficiency of structures with a biological or equivalent membrane or envelope, such as cells into eukaryotic cells than as described for the methods in the prior art. Accordingly, in a preferred embodiment the method for providing cells with a desired phenotypic characteristic by introduction (uptake) of structures provided with a biological or equivalent membrane, or envelope and associated with a PAR binding ligand into said cell as described herein is provided, wherein the uptake efficiency of the structures respective cells by the eukaryotic cells is higher than 5 %, in particular about 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39% or about 40% or in any range formed by these values, e.g., between 6% and 10% or between 10% and 20% or between 6 and 30%.

The method of the present invention can be used to provide a (host) cell with a bandwidth of different phenotypic characteristics. Preferably the provided phenotypic characteristic is a gain of a phenotypic characteristic not present in the initial cell before the uptake of the structure or an increase or decrease of a phenotypic characteristic already present in the initial cell.

In one embodiment, the provided phenotypic characteristic is an effect on (an alteration of) production or consumption of a molecule. Provision of a desired phenotypic characteristic means in this context that due to the subjection of a cell to the method of the present invention this cell starts with the production or consumption of a molecule, wherein it was not produced or consumed by the cell before the method was performed. Under an alteration, however, also a change in the amount of production or consumption is understood, *i.e.* if a product was produced on consumed by the cell before the method was performed and the amount thereof changed (raised or decreased) after the cell was subjected to the method described herein. As

an example, after the introduction of the structure by the method of the present invention into the host cell, this cell can start to produce a gas, such as oxygen or carbon dioxide or reduce its requirement for oxygen supply from outside (or its oxygen consumption respectively), start to produce a given peptide or protein, wherein the cell was not producing or consuming any of these before the method was performed. Similarly, the method of the present invention can be used to amend a cell in that way that the cell which was already producing or consuming a gas or a protein, changes the level of production or consumption after being subjected to the method of the present invention as described herein. As to the reduction of oxygen consumption, this reduction is primarily understood as a reduction of requirement for oxygen supply, due to the own production by the structure introduced into the host cell. Further examples of molecules, the production, consumption or modification of which can be altered in the host cells subjected to the methods described herein besides these already mentioned above (*e.g.*, gases) can be selected from the group consisting of carbohydrates (including sugars), proteins (including polypeptides and peptides), lipids, nucleic acids, anorganic ions, glycoproteins and other glycoconjugates .

Furthermore, the method of the present invention can be also performed to provide a phenotypic characteristic by a change of a response of the host cell to an added substance, *e.g.*, to a drug or toxin or an effect on the ability of the host cell to grow or to die in an environment or an observable alteration of a cultural, behavioral or morphological characteristic of the host cell or induction of death of a cell.

Both prokaryotic and eukaryotic cells can be used in the methods of the present invention as host cells. In a preferred embodiment the host cell is a eukaryotic cell, preferably a mammalian cell and mostly preferred a human cell.

As already discussed above, the phenotypic characteristic provided by the method of the present invention can be induction or amendment of the level of production of a gas by the host cell which took up a structure because of the subjection under the method of the present invention, or a reduced consumption of a gas. Accordingly, in one embodiment, the phenotypic characteristic is production of oxygen or reduced oxygen consumption by or in the host cell.
In this connection, whenever it is indicated that the phenotypic characteristic concerns an induction of production, amendment (increase or decrease) of the level of production or amendment (increase or decrease) in the level of consumption of a product, despite the

indication in singular, the desired phenotypic characteristic can also comprise corresponding effects on several molecules or products, *e.g.*, the induction or change of the production or consumption level of several gases or molecules, *e.g.*, the production of two (or more) different gases or polypeptides can be induced due to the subjection of a cell to the method of the present invention. Also, the phenotypic characteristics do not have to be all the same in kind, *e.g.*, they do not all have to be an induction or increased level of a production of any product, but the phenotypic characteristics provided to a host cell by the method of the present invention can be, *e.g.*, an induction or increased level of production of a gas and a reduction of consumption of the same or another gas or of another product by the host cell. Another not limiting example would be the induction or increased level of production of a polypeptide and a reduction of production or reduction of consumption of a gas or a carbohydrate such as a sugar or another product.

As to the cells which can be altered by the method of the present invention, preferably the host cell is a human cell. Several human cell types can be subjected to the method of the present invention, in a preferred embodiment the method described herein above and below is performed, wherein the host cell is a human keratinocyte.

As to the structures which are introduced by the method of the present invention into the cells, or host cells respectively, preferably the method described herein above and below is performed, wherein the structure is a cyanobacterium.

In a preferred embodiment the method of the present invention is provided, wherein the (host) cell is a human keratinocyte, the structure is a cyanobacterium and wherein the phenotypic characteristic is production of oxygen or reduced oxygen consumption by or in the human keratinocyte.

The present invention further relates to a cell which is altered in a phenotypic characteristic compared with an initial host cell obtainable by the method of the present invention as described hereinabove. The term "initial host cell" as used herein refers to a host cell, which has not been subjected to the method of the present invention.

Since as already mentioned current approaches for the treatment of chronic, non-healing wounds exhibit insufficient success rates, are highly expensive, or are still in its infancy and possibly associated with numerous side effects, it is mandatory to develop new strategies. In

contrast to other reported treatments, the present invention aims at specifically overcoming hypoxic wound conditions caused by macro- and microangiopathy. Numerous studies have demonstrated that hypoxia and insufficient recovery from oxygen deprivation are major factors responsible for breakdown and necrosis of human skin tissue. As a consequence, current scientific therapies aiming at the enhancement of cell proliferation in wounds might be functional in young and healthy individuals but would probably fail in older patients, or for those suffering from chronic metabolic diseases. In the elderly, the reduced oxygen supply to the wound tissue is the central cause for insufficient cellular regenerative processes that are associated with high metabolic energy conversion rate.

The concept of one aspect of the present invention is to address the cellular oxygen demand by incorporating photosynthetic, oxygen-producing cyanobacteria into skin cells (keratinocytes) to generate neo-endosymbiotic, photosynthetic keratinocytes (Fig. 1). Neo-endosymbiotic keratinocyte grafts on non-healing wounds would autonomously produce oxygen upon light exposure and thereby circumvent local hypoxia followed by enhanced wound closure. The development of a neo-endosymbiotic mammalian unit that resembles the evolutionary fusion of plant and bacterial cells represents a completely new approach to tackle human disease.

Accordingly, in a further aspect, the present invention also relates to a composition or kit comprising the host cell provided with a desired phenotypic characteristic by the method described herein above and below, wherein the composition is a pharmaceutic composition comprising a pharmaceutically acceptable carrier.

In order to have the desired therapeutic effect the composition of the present invention is preferably contacted with the skin area to be treated. Accordingly, in a preferred embodiment of the present invention the composition is designed for topical, intralesional, intraepithelial, intra-epidermal, intra-cutaneous or subcutaneous administration into or preferably onto the skin. Further grafting modalities comprise supra- and sub-fascial, supra-muscular or any other soft tissue surface placement of the composition.

In addition, the kits may include instructional materials containing directions (*e.g.*, protocols) for the practice of the methods of this invention or the composition can be provided with such. The present invention also relates to the cell of obtainable by the methods described herein or the pharmaceutical composition for use in the treatment of wounds. In particular, the present

invention relates to a eukaryotic cell which has been provided by the method described herein with a phenotypic characteristic, wherein the phenotypic characteristic is production of oxygen or reduced oxygen consumption compared with a corresponding wild type cell (or initial host cell). In a preferred embodiment that cell is a keratinocyte, mostly preferred a human keratinocyte. Furthermore, preferably the structure introduced in that cell by the method of the present invention is preferably a cyanobacterium

In a further aspect, the present invention also relates to a use of a structure provided with a biological or equivalent membrane or envelope or equivalent conjugated with a PAR binding ligand as described herein for the generation of an endosymbiotic cell by allowing that the structure conjugated with a PAR binding ligand is taken up by a host cell expressing a protease activated receptor (PAR) on its surface, wherein the structure is a cell and preferably wherein the host cell is a eukaryotic cell.

In another aspect the present invention further relates to a use of a PAR binding ligand for the generation of an endosymbiotic cell. In this use preferably a PAR binding ligand is used as defined hereinabove in the context of the method of the present invention for providing a cell with a desired phenotypic characteristic, preferably wherein by allowing that a structure as described before which is conjugated with or associated to such a PAR binding ligand is taken up by a host cell, wherein the host cell expresses a PAR on its surface. Preferably the structure is a cell for this intended use, further preferred the host cell is a eukaryotic cell.

Definitions and embodiments:

It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a polypeptide," is understood to represent one or more polypeptides. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

The term "about" as used herein defines a possible deviation from the concerned value in the range of 1%-30%, in particular of 1%-20%, in particular of 1%-10%, in particular of 1%-5%, in particular of 1%-2% of the value as defined without usage of this term.

Unless stated otherwise, the terms "disorder" and "disease" are used interchangeably herein.

By "subject" or "individual" or "animal" or "patient" or "mammal", is meant any subject, particularly an avian subject, e.g., falcons, buzzards, hawks and eagles or a mammalian subject,

e.g., animals, such as pets and farm animals, in particular cats, dogs, cattle, horses, sheep, goats or a human patient, for whom diagnosis, prognosis, prevention, or therapy is desired.

Polynucleotides:

The term "polynucleotide" as used herein is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA) or plasmid DNA (pDNA). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)). The term "nucleic acid" or "double-stranded nucleic acid"refers to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a polynucleotide, comprising preferably the sequence encoding at least one miRNA molecule of the present invention. By "isolated" nucleic acid or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide encoding at least one miRNA or an antibody contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of polynucleotides of the present invention. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

As used herein, a "coding region" is a portion of nucleic acid which consists of codons translated into amino acids or comprising the sequence precursor of a miRNA molecule of the present invention. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. The coding region may be a nucleotide sequence coding for an amino acid sequence or a functional RNA, such as tRNA, rRNA, catalytic RNA, miRNA, siRNA and antisense RNA. A coding region may also be an mRNA or cDNA corresponding to the coding regions (*e.g.*, exons and miRNA) optionally comprising 5′- or 3′-untranslated sequences linked thereto. A coding region may also be an amplified nucleic acid molecule

produced in vitro comprising all or a part of the coding region and/or 5′- or 3′-untranslated sequences linked thereto.

Two or more coding regions of the present invention can be present in a single polynucleotide construct, *e.g.*, on a single vector, or in separate polynucleotide constructs, *e.g.*, on separate (different) vectors. Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions, *e.g.*, a single vector may separately encode an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region. In addition, a vector, polynucleotide, or nucleic acid of the invention may encode heterologous coding regions, either fused or unfused to a nucleic acid encoding a binding molecule, an antibody, or fragment, variant, or derivative thereof. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain.

In certain embodiments, the polynucleotide or nucleic acid is DNA. In the case of DNA, a polynucleotide comprising a nucleic acid which encodes a miRNA or polypeptide normally may include a promoter and/or other transcription or translation control elements operably associated with one or more coding regions. An operable association exists when a coding region for a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a miRNA/polypeptide coding region and a promoter associated therewith) are "operably associated" or "operably linked" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a miRNA/polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription. Suitable promoters and other transcription control regions are disclosed herein.

A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (the immediate early promoter, in conjunction with intron-A), simian virus 40 (the early promoter), and retroviruses (such as Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit ß-globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (*e.g.*, promoters inducible by interferons or interleukins).

Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence).

Treatment and drugs:

As used herein, the terms "treat" or "treatment" refer to therapeutic or cosmetic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the development of an autoimmune and/or autoinflammatory disease or an undesired amendment in particular of the appearance of the skin, hairs, feathers, nails, claws, beaks or hoofs. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease or the undesired appearance, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression or of the progression of the undesired appearance, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the manifestation of the condition or disorder is to be prevented.

If not stated otherwise the term "drug," "medicine," or "medicament" are used interchangeably herein and shall include but are not limited to all (A) articles, medicines and preparations for internal or external use, and any substance or mixture of substances intended to be used for

diagnosis, cure, mitigation, treatment, or prevention of disease of either man or other animals; and (B) articles, medicines and preparations (other than food) intended to affect the structure or any function of the body of man or other animals; and (C) articles intended for use as a component of any article specified in clause (A) and (B). The term "drug," "medicine," or "medicament" shall include the complete formula of the preparation intended for use in either man or other animals containing one or more "agents," "compounds", "substances" or "(chemical) compositions" as and in some other context also other pharmaceutically inactive excipients as fillers, disintegrants, lubricants, glidants, binders or ensuring easy transport, disintegration, disaggregation, dissolution and biological availability of the "drug," "medicine," or "medicament" at an intended target location within the body of man or other animals, *e.g.*, at the skin, in the stomach or the intestine. The terms "agent," "compound", or "substance" are used interchangeably herein and shall include, in a more particular context, but are not limited to all pharmacologically active agents, *i.e.* agents that induce a desired biological or pharmacological effect or are investigated or tested for the capability of inducing such a possible pharmacological effect by the methods of the present invention.

Pharmaceutical carriers:

Pharmaceutically acceptable carriers and administration routes can be taken from corresponding literature known to the person skilled in the art. The pharmaceutical compositions of the present invention can be formulated according to methods well known in the art; see for example Remington: The Science and Practice of Pharmacy (2000) by the University of Sciences in Philadelphia, ISBN 0-683-306472, Vaccine Protocols.2nd Edition by Robinson *et. al.*, Humana Press, Totowa, New Jersey, USA, 2003; Banga, Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems. 2nd Edition by Taylor and Francis. (2006), ISBN: 0-8493-1630-8. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well-known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways. Examples include administering a composition containing a pharmaceutically acceptable carrier via oral, intranasal, rectal, topical, intraperitoneal, intravenous, intramuscular, subcutaneous, subdermal, transdermal, intrathecal, endoscopic, intraarticular and intracranial methods and any other kinds of injections or placement. Aerosol formulations such as nasal spray

formulations include purified aqueous or other solutions of the active agent with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes. Pharmaceutical compositions for oral administration, such as altered cells are also envisaged in the present invention. Such oral formulations may be in tablet, capsule, powder, gel, liquid, semi-solid form, or any other applicable form, such as used, in general for Advanced Therapy Medicinal Products (ATMPs). In such ATMP-formulations, the cells may be, e.g., provided in hydrogel, matrix or tissue associated forms, combined with culture media, detergents, sera, enzymes, hormones, cryopreservatives, stabilizing agens and/or can be in addition comprised in a drug-device combination. A tablet may comprise a solid carrier, such as gelatin or an adjuvant. Formulations for rectal or vaginal administration may be presented as a suppository or tamponade with a suitable carrier; see also O'Hagan *et. al.*, Nature Reviews, Drug Discovery 2(9) (2003), 727- 735. Further guidance regarding formulations that are suitable for various types of administration can be found in Remington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985) and corresponding updates. For a brief review of methods for drug delivery see Langer, Science 249 (1990), 1527-1533. "Pharmaceutical acceptable" refers to a medium that do no not produce an allergic or similar untoward reaction when administered to an animal or a human.

Expression:

The term "expression" as used herein refers to a process by which a gene produces a biochemical, for example, a RNA, a miRNA or polypeptide. The process includes any manifestation of the functional presence of the gene within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression. It includes without limitation transcription of the gene into messenger RNA (mRNA), transfer RNA (tRNA), small hairpin RNA (shRNA), small interfering RNA (siRNA) or any other RNA product, and the translation of such mRNA into polypeptide(s). If the final desired product is a biochemical, expression includes the creation of that biochemical and any precursors. Expression of a gene produces a "gene product." As used herein, a gene product can be either a nucleic acid, *e.g.*, micro RNA (miRNA), a messenger RNA produced by transcription of a gene, or a polypeptide which is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation, or polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, proteolytic cleavage, and the like.

Cyanobacteria:

Cyanobacteria or blue green algae are prokaryotic photosynthetic, gram negative bacteria that are one of the oldest cell types on earth (Badger & Price, 2003). They are assumed to have played a significant role in evolutionary historic events, such as the Great Oxidation Event (GOE) (Rippka, *et. al.*, 1979), the evolution of endosymbiosis and eukaryotic organisms (Sagan, 1967). Due to the autotrophic mode of nutrition, cyanobacteria exist in a wide variety of habitats in different geographic areas, from arctic regions to hot springs which reflects on their genotypic and phenotypic variations (Chorus & Bartram, 1999). All photosynthetic organisms convert light energy into chemical energy required to synthesize sugars from CO_2 and H_2O (Arnon, 1959). The photoautotrophs absorb light energy using their photosynthetic apparatus consisting of multiprotein complexes. These include photosystem II (PSII), cytochrome b6f (Cyt b6f), photosystem I (PSI) and ATP synthase which are common among the cyanobacteria and algae or higher plants. The light harvesting complex of PSII called phycobilisome (PBS) is unique to cyanobacteria. PBS consist of a core allophycocyanin (APC) and form rods of macromolecular complexes around it (Bryant, *et. al.*, 1979). The rods contain accessory photosynthetic pigments phycobiliproteins, which covalently bind to the light-absorbing chromophores phycobilins. The light-dependent reactions further lead to ATP production *via* photosynthetic electron transport chain and oxygen is released as a by-product. In the light-independent phase, CO_2 is fixed in the Calvin-Benson cycle resulting in the formation of monosaccharides (Pheiss & Kosuge, 1970).

Cyanobacteria contain a unique cell wall organization, wherein structural elements of Gram-positive (peptidoglycan layer) and Gram-negative (contain outer membrane) are combined (Woitzik, *et. al.*, 1988). The cytoplasm of cyanobacteria is surrounded by a cell membrane followed by peptidoglycan layer, an outer membrane and an external mucilaginous layer.

Considering the structural composition of cyanobacteria, the present application uses *Synechococcus elongatus* strain PCC 7942 for investigating their targeted cellular uptake by keratinocytes. *S. elongatus* PCC 7942 are approximately 2-5 µm in length and their doubling time is about 7-8 hours. It is a freshwater strain and was isolated from a lake in California, USA in 1973 (Rippka, *et. al.*, 1979). Although the structural and chemical composition of cell wall and external layers of cyanobacteria *S. elongatus* PCC 7942 largely remain unidentified, similar research has been done on few other strains. For example, studies on *Synechocystis*

PCC 6714 suggest that the external layer consists of glycoproteins and polysaccharide whereas the outer membrane is composed of lipopolysaccharide, proteins, lipids and carotenoids (Jürgens & Weckesser, 1985). The structural elements of cyanobacterial outer layers contain various hexoses (fructose, galactose, glucose and mannose), pentoses (arabinose, ribose and xylose) and deoxyhexoses (fucose and rhamnose), as well as the acidic sugars, glucuronic and galacturonic acid (Kehr & Dittmann, 2015). Some of the components have free carboxyl groups, which can be utilised as target structures for the covalent conjugation of P8 molecule to the surface of S. elongatus PCC7942.

Coupling reagents:

The approach followed in the exemplary experiments described herein included amide coupling reactions between the carboxyl groups on the surface of *S. elongatus* PCC 7942 and primary amine group provided by P8. A carboxylic acid and an amine need to react at temperatures above 200°C to form an amide bond (Jursic & Zdravkovski, 1993). Since an amide bond does not spontaneously form at physiological conditions, chemical reagents were used for cross-linking purpose.

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) was used to activate carboxyl groups present on the surface of *S. elongatus* PCC 7942 to further react with amines. EDC is a water-soluble compound and it does not become a part of the final amide bond between the target molecules (Mattson, *et. al.*, 1993), the cyanobacteria with P8 in the case described herein. Carbodiimides (EDC) are commonly used as coupling reagents, which react with carboxylic acids to generate intermediate compounds that contain good leaving groups such as active esters (Bodanszky, 1979). Active esters are highly susceptible to nucleophilic substituents like amino groups to further form amide bonds with the original carboxyl groups. In the first step of amide coupling reaction, the carboxylic acid reacts with EDC to form O-acylisourea intermediate ester, which in the next step spontaneously reacts with a primary amine to form an amide bond. Addition of N-hydroxysuccinimide (NHS) to the reaction mixture in the second step increases the coupling efficiency and forms NHS-ester that is more stable to amine-reactivity than the *O*-acylisourea intermediate. This reaction also forms a urea-derivative by-product. The final step replaces the NHS moiety from the intermediate NHS-ester with a primary amine group in the vicinity and produces an amide linkage between the carboxyl and amine groups from target substances (Mattson, *et. al.*, 1993).

Nevertheless, the method of the present invention can be also performed by conjugation of PAR ligands to other functional groups or atoms besides a carboxyl groups. Preferably the functional group or atom is selected for the conjugation with the PAR ligand which is characterized by its nucleophilic (Lewis base) or electrophilic (Lewis acid) property.

Accordingly, the method of the present invention is provided herewith wherein the PAR ligand is conjugated to a functional group of a molecule, which is component of the structure to be introduced into a host cell (preferably, part of the surface of the structure), wherein the functional group is selected from the group consisting of: carboxylic acid, carboxylic ester, azide, alkene, alkyne, amine, alcohol, aldehyde, nitrile, thiol, epoxide.

Cell membranes, envelopes and equivalents

Envelope – the term envelope as used herein is directed to outermost layers of viruses and pro- or eukaryotic organisms, such as bacteria, in particular cyanobacteria and to layers comprising the components of these layers. A viral envelope or equivalent comprises or consist of one or more from the group consisting of: phospholipids, proteins and some also glycoproteins. A pro- or eukaryotic envelope or cell wall comprises or consist of one or more from the group consisting of: lipopolysaccharides, proteins, pepditoglycans and phospholipids. Preferred structures as referred to herein comprising or surrounded by an envelope are therefore prokaryotic organisms, viruses and virus-like particles. The term surrounded means in this context, that the surface of the structure comprises or consists of an envelope.

The term cell membrane as used herein refers to a membrane consisting of a lipid bilayer preferably comprising, consisting or substantially consisting of phospholipids or equivalents thereof forming membranes and comprising or consisting of lipids. Optionally the cell membrane or equivalent thereof comprises in addition one or more from the group consisting of: cholesterols, proteins, carbohydrates and fibers or compositions (or conjugates) thereof. Preferred structures as referred to herein comprising or surrounded by a cell membrane are therefore cells, microvesicles, vesicles and exosomes or other structures indicated already above. The term surrounded means in this context, that the surface of the structure comprises or consists of a cell membrane consisting of phospholipids alone or comprising in addition anyone or more of cholesterols, proteins, carbohydrates, fibers.

Under equivalents of biological membranes or structures structures are understood herein, which show similar properties as a biological cell membrane or envelope, in particular are suitable to enclose biological entities, such as nucleic acids and/or polypeptides, peptides, however, are produced synthetically. Basically, all polymers representing the current state of the art, which can be used for encapsulation of therapeutic or diagnostic cargo, can function as equivalent in the sense of this invention. In one embodiment equivalents comprise or consist only one of the aforementioned components of a cell membrane or envelope, *e.g.*, only phospholipids. In one embodiment equivalents of cell membranes or envelopes consist or comprise of one or more molecules selected from the group consisting of: polymers (including alginate, cellulose, chitin, and thermoplastic polymers), crosslinked proteins, lipid membranes, polymer-lipid complexes, albumin, liposomes, and others.

The term "cell" or "host cell" is used herein to denote any cell, wherein any foreign or structure has been introduced or is intended to be introduced. In its broadest sense, "host cell" is used to denote a cell which is intended to or has been manipulated by introduction of a structure as defined herein by the method of the present invention.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the materials, methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries and databases, using for example electronic devices. For example, the public database "Medline" may be utilized, which is hosted by the National Center for Biotechnology Information and/or the National Library of Medicine at the National Institutes of Health. Further databases and web addresses, such as those of the European Bioinformatics Institute (EBI), which is part of the European Molecular Biology Laboratory (EMBL) are known to the person skilled in the art and can also be obtained using internet search engines. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The above disclosure generally describes the present invention. Unless otherwise stated, a term as used herein is given the definition as provided in the Oxford Dictionary of Biochemistry and Molecular Biology, Oxford University Press, 1997, revised 2000 and reprinted 2003, ISBN 0 19 850673 2. Several documents are cited throughout the text of this specification. Full

bibliographic citations may be found at the end of the specification immediately preceding the claims. The contents of all cited references (including literature references, issued patents, published patent applications as cited throughout this application and manufacturer's specifications, instructions, etc.) are hereby expressly incorporated by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLES

Materials and methods

1.1 Culturing of cyanobacteria

1.1.1. Medium preparation for culturing cyanobacteria

The cyanobacteria strain which was used in this work was *Synechococcus elongatus* PCC 7942. This strain was originally isolated from freshwater lake in California US (Golden, 2018). It was acquired for research purpose from The Pasteur Culture Collection of Cyanobacteria (Paris, France). PCC 7942 was cultivated in the enriched artificial seawater medium (ESAW) (Berges, *et. al.*, 2001). ESAW formulation was provided by the Provasoli-Guillard National Center for Marine Algae and Microbiota (East Boothbay, ME, USA). According to the formulation, (Refer appendix table 7.1-7.6 for stock preparation) stock of anhydrous salts (salt I) was dissolved in approximately 500 mL of Milli-Q water. In another flask, stock of hydrated salts (salt II) was dissolved in approximately 300 mL of Milli-Q water. These two salt solutions salt I and II were mixed together in a 2 L flask. Major nutrients including $NaNO_3$ (1 mL L^{-1}), NaH_2PO_4 (1 mL L^{-1}) and Na_2SiO_3 (2 mL L^{-1}), iron-EDTA (1 mL L^{-1}), trace metals (1 mL L^{-1}) and vitamin solutions (1 mL L^{-1}) were added and the volume was filled up to 1 L with Milli-Q water to form ESAW medium with 100 % osmolarity or osmic concentration. Previously, the cyanobacterial strain *S. elongatus* PCC 7942 was adapted to grow in 30 % osmolarity of ESAW medium. Hence 30 % ESAW was prepared from 100 % ESAW by reducing salt concentration to 30 % while keeping all other nutrient and vitamin contents according to the recipe formulated by Berges, Franklin, & Harrison, 2001. Additionally, 10 mM TRIS was added and the pH was set to 7.4. ESAW 30 % medium was sterile filtered through a 0.2 µm pore filter and stored at 4°C.

1.1.2 Cultivation of cyanobacteria

Cultures were established by collecting the cryotubes containing cyanobacteria *Synechococcus elongatus* PCC 7942 from liquid nitrogen storage. They were thawed in the dark for about 30 minutes to gradually acclimatise the cells to *in vitro* culture conditions. 1 mL content of each cryotube was transferred to a Falcon tube and 10 mL 30% ESAW medium was added to it. This tube was centrifuged for 15 minutes at 6000 rpm at room temperature to completely remove dimethyl sulfoxide (DMSO) from the sample. Supernatant was discarded and cyanobacterial pellet was resuspended into 40 mL fresh 30% ESAW medium under sterile conditions in a 100 mL culture flask. The flask was incubated in a phytotron at 30°C at 12:12 diurnal cycle at ca. 100 µmol photons m^{-2} s^{-1}.

Every week for culture maintenance, cell count of cyanobacteria was checked using flow cytometry (Refer section 3.7) and sub-culturing was performed in case the density of cultures reached more than 40 million cells per ml. 400 µL cell culture was inoculated into 40 mL of fresh 30 % ESAW for sub-culturing, under sterile conditions and incubated in a phytotron at 30°C at 12:12 diurnal cycle at ca. 100 µmol photons m^{-2} s^{-}.

1.1.3 Cyanobacteria culture cryopreservation

After cyanobacteria PCC 7942 cultures had reached exponential growth phase, some of the aliquots were cryopreserved and stored in liquid nitrogen. 80 mL densely growing cell cultures were aliquoted into two 50 mL Falcon tubes. Tubes were centrifuged at 5000 rpm for 15 minutes at room temperature. Supernatant was discarded and pellets were resuspended in fresh 9 ml 30 % ESAW medium. 1 mL culture was aliquoted in 9 cryotubes and 18 µL 1.8 % (v/v) DMSO was added to each of the cryotubes. Cultures were frozen using a freezing container with isopropanol to gradually decrease temperature at cooling rate of -1 ° C / minute, and subsequently transferred to liquid nitrogen for long term storage.

2. Culturing HaCaT cells (human keratinocyte cell line)

2.1. Thawing of frozen HaCaT cells

HaCaT, an aneuploid immortal keratinocyte cell line from adult human skin (Boukamp, *et. al.*, 1988) was used in this study. Cryopreserved aliquots of HaCaT cells were thawed for about 30 minutes at room temperature. 1 ml aliquots of thawed HaCaT cells were transferred to a Falcon tube filled with 9 mL freshly prepared DMEM + 10 % (v/v) FCS, respectively. Tubes were centrifuged at 900 rpm for 5 minutes. After centrifugation, the supernatant was carefully

removed, and each cell pellet was resuspended in 1 mL DMEM + 10 % FCS. Meanwhile, 10 cm culture plates were prepared by adding 9 mL DMEM + 10 % FCS and 10 µL 50 µg/mL Gentamycin to each plate. 1 mL cell suspension was transferred to each 10 cm culture plate and incubated at 37°C, 5 % CO_2.

2.2 Cultivation of HaCaT cells

HaCaT cell cultures were maintained every week by replacing the old media with fresh DMEM + 10 % or 5 % (v/v) FCS depending upon the cellular growth rate and confluency of the cultures. Old media was removed, and cells were washed thrice with 10 mL of prewarmed PBS. 10 mL of fresh DMEM + 10 % or 5 % (v/v) FCS were added to the washed cells. 10 µL Gentamycin was added to the medium to reach a final concentration of 50 µg/mL. Cell culture plates were incubated at 37°C, 5 % CO_2.

HaCaT culture plates were split whenever 80-90% confluency was reached to prevent cellular overgrowth. The existing culture medium was removed and cells were thoroughly washed thrice with 10 mL PBS. 1 mL 0.05 % (v/v) trypsin was added to the washed cells, spread evenly and incubated for about 10 minutes at 37°C. 9 mL DMEM + 10% (v/v) FCS was added to the plate to inactivate the trypsin. Cells from one culture plate were transferred to a Falcon tube and centrifuged for 5 minutes at 1000 rpm. Subsequently, the supernatant was removed and the cell pellet was resuspended in 1 mL fresh DMEM + 10% (v/v) FCS. Resuspended cells were distributed in culture plates with medium and antibiotics and in an incubator at 37°C, 5 % CO_2 for further experiments (see section 3.2.1).

2.3 Cryopreservation of HaCaT cells

80-100% confluent HaCaT culture plates were chosen for cryopreservation to return the cell stocks to liquid nitrogen storage. HaCaT cell cultures were trypsinised using a 0.05 % (v/v) trypsin solution. Cells were transferred to a Falcon tube and centrifuged for 5 minutes at 1000 rpm. Cell pellets were resuspended in 1 mL DMEM + 20% (v/v) FCS. 100 µL DMSO was pipetted to each cryotube. 900 µL cell suspension was added and tubes were gently inverted. Cultures were frozen using a freezing container with isopropanol to gradually decrease temperature at cooling rate of -1 ° C / minute, and subsequently transferred to liquid nitrogen for long term storage.

3. Isolation and culturing of primary human keratinocytes

3.1 Isolation of primary keratinocytes from human skin tissue

Primary keratinocytes were obtained from human foreskin samples provided by Professor Hess Children's Hospital in Bremen, Germany. Under sterile conditions, foreskin samples were transferred to a petri dish without any liquid. Upper skin and lower skin were separated from the tissue samples and were chopped into fine pieces in 2 separate petri plates. Cut pieces of skin tissue were transferred to 25 cm^2 cell culture flasks. 6 mL 0.1% (v/v) collagenase solution in PBS was added to each flask while fully covering the tissue pieces with the solution. 60 µL 1% (v/v) Penicillin-Streptomycin solution was added to each flask and the flasks were incubated overnight at 37°C, 5% CO_2.

Next day, tissue pieces in collagenase were observed under a microscope for the separation of cells. Supernatant from each flask was transferred into a Falcon tube and the remaining tissue clumps were homogenized. Equal volume of TCC199 medium containing 20% (v/v) FCS was added to the supernatant in the Falcon tube to inactivate the collagenase. Samples were centrifuged at 1100 rpm for 10 minutes at room temperature and the supernatant was carefully removed keeping about 1 mL of it in the tubes. Cell pellets were resuspended and cell suspensions were transferred back to the cell culture flasks. 6 mL KGM2 medium, 60 µL 1% (v/v) Penicillin-Streptomycin solution were added to each flask and the flasks were incubated at 37°C, 5% CO_2. After 5-6 days, cultures were observed under microscope for potential contamination or growing cells and were accordingly discarded or processed further.

3.2 Culturing of isolated primary human keratinocytes

Every week the KGM2 medium was replaced in each flask containing primary keratinocyte. Entire medium from the flask was transferred into a Falcon tube without disturbing the adherend keratinocytes. The medium along with suspending keratinocytes was centrifuged at 900 rpm for 5 minutes and supernatant was discarded. Cells were resuspended in 1 mL fesh KGM2 medium and were transferred back into the same flask. Additional 5 mL KGM2 medium and 60 µL 1% v/v Penicillin-Streptomycin solution were added. Flasks were incubated at 37°C, 5 % CO_2.

The isolated cells were split when confluency of 80-90 % was reached. Cells were detached using 1 mL, 0.05 % (v/v) trypsin solution for 10 minutes at 37 °C and 5% CO_2. 4.5 mL DMEM

+ 20% (v/v) FCS was added to the flask. Cell suspension was transferred to a 50 mL Falcon tube, centrifuged at 900 rpm for 5 min and supernatant was subsequently discarded. Cell pellets were resuspended in fresh KGM2 medium and cells were counted by flow cytometry. New flasks were inoculated by seeding 1-2 million cells per flask (Refer sections 3.3 and 3.6). Immunocytochemical staining and epi-fluorescence microscopy was performed to characterise isolated cells (Refer sections 3.5 and 3.8).

3.3 Cryopreservation of primary human keratinocytes

Cryopreservation of primary keratinocytes was performed as soon as a confluency of about 90 % was reached. To freeze the cells, KGM2 medium was removed and cells were washed thrice with 5 mL PBS. Cells were detached using 0.05 % (v/v) trypsin solution and incubated for 10 min at 37°C. Cell suspension was centrifuged for 10 minutes at 900 rpm and the supernatant was discarded (section 3.3). The cell pellet was resuspended in Cryo SFM and transferred into cryotubes. Cells were frozen using a freezing container with isopropanol to gradually decrease temperature at cooling rate of -1 ° C / minute, and subsequently transferred to liquid nitrogen for long term storage.

3.4 Cytosol extraction from cultured HaCaT cells

The capacity of the cyanobacterial strain PCC7942 to adapt and survive within the actual cytosol extracted from HaCaT cells was investigated. HaCaT cells were detached from culture plate using a 0.05 % (v/v) trypsin solution and incubated for 10 min at 37°C. Cell suspension was centrifuged for 10 minutes at 900 rpm and the supernatant was discarded. Cell pellets were resuspended in 5 mL phenol red-free DMEM. Flow cytometry was separately performed using 1 mL samples of both cyanobacteria and HaCaT cells to determine their cell numbers respectively (section 3.7). HaCaT cell suspension containing approx. 1 million cells were diluted in 1 mL PBS. Cells were lysed mechanically using a homogenizer in aliquots of 100 µL, for 4 cycles of 10 sec lysis followed by breaks of 10 sec, at maximum instrumental power on ice to reduce adverse effects of heat generated during cell lysis. Effective cell lysis was confirmed by flow cytometry. The cell lysate was centrifuged at 14,000 rpm for 10 minutes to separate larger cell debris.

1 million cyanobacteria PCC 7942 were inoculated in cytosol extracts isolated from 1 million HaCaT cells. Incubations were carried out for up to 48 hours within a phytotron at 30°C at a

12:12 diurnal cycle at ca. 100 µmol photons m^{-2} s^{-1}. Vitality of cyanobacteria was analysed by flow cytometry (section 3.7) after incubation time points of 3, 24 and 48 hours. Control samples were set up using 30% ESAW medium instead of cytosol extracts. Data was collected by CyFlow Cube 16 software and analysed using FCS Express 7.

3.5 Chemical conjugation of P8 to surface molecules of cyanobacteria PCC7942

Synechococcus sp. have an external mucilaginous layer around the cells which is made up of glycoproteins and polysaccharides (Hoiczyk & Hansel, 2000). By targeting these surface molecules, the cyanobacteria *S. elongatus* PCC 7942 were biochemically modified for subsequent attachment of P8 molecules. P8 linker molecule was generated for performing the method of the present invention that contains a fluorophore, a linker subunit having a free primary amine group and a hexapeptide capable of binding and activating PAR2, separated by a spacer element as described in detail in WO 2019/057988, in Examples 1 and 2 on pages 12 to 17, wherein the design of novel PAR ligands is described in Example 1 on pages 12 to 13 and the synthesis of the novel PAR2 agonist P8 is described in Example 2 on pages 13 to 17, the content of which experimental description is herein incorporated in its entirety by reference.

Coupling of the cyanobacterium has been performed in principle similar as described in Example 5 on page 20 of WO 2019/057988, the content of which experimental description is incorporated herein in its entirety by reference with some amendments, which are pointed out below.

Coupling reagents EDC (N′-ethylcarbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) were used for the attachment of P8 to surface molecules of cyanobacteria PCC 7942. EDC activates carboxyl groups and forms unstable O-acylisourea intermediate esters which spontaneously react with primary amines to form amide bonds. Addition of NHS to the reaction mixture increases the coupling efficiency and stability of amine reactive intermediate esters (Mattson, *et. al.*, 1993).

In brief, cyanobacteria were aliquoted under sterile conditions and density was determined using flow cytometry (see section 3.7 below). A reaction mixture was prepared in a Falcon tube by aliquoting 1 million cells of *S. elongatus* PCC 7942 and adding various components at their final concentrations as follows: 20 µM NHS, 10 µM EDC, 1 mM P8 linker molecule (instead

of 500mM NHS, 10 mM EDC and 29 mM P8 as used in WO 2019/057988). PBS was added to make up the volume to 1 mL and the reaction mixture was incubated on a rotation wheel, in the dark, at room temperature for 1 hour.

This following step is entirely new in this protocol. The reason for the addition of glycine is to utilise its C-alpha amino group for the reaction with remaining NHS-activated carboxylgroups still present on the surface of cyanobacteria (deactivation). It should be noted that remaining NHS-activated carboxylgroups could in theory also react with amino groups of other surface molecules of present cyanobacteria as a result of trans-interactions between two or more cyanobacteria. The result of that could be a "clumping" of cyanobacteria.

Therefore, to avoid such clumping, after 1 h of incubation, a glycine solution was added to the reaction mixture to yield a final concentration of 1 mM and incubated under same conditions for additional 15 minutes.

Afterwards, reaction tubes were centrifuged at 5000 rpm for 10 minutes and the supernatant was removed. Cyanobacterial pellet was resuspended in 1 mL fresh PBS and samples were analysed by flow cytometry.

3.6 Endocytosis assay

The interactions of surface modified cyanobacteria with human keratinocytes were studied by performing an endocytosis assay. Endocytosis assay was performed on HaCaT cells or primary human keratinocytes in 4 steps as follows:

i) Keratinocyte seeding:

a) HaCaT cells: HaCaT cells were used as the mammalian cell material for establishing the endocytosis assay. Cells were detached from the plates using 1 mL, 0.05 % (v/v) trypsin solution. Cells were centrifuged at 900 rpm for 5 minutes and supernatant was discarded. Pellets were resuspended in phenol red-free DMEM and cell density was determined by flow cytometry (section 3.2, 3.3 and 3.7). Approximately 90,000 cells/cm^2 were seeded on a coverslip in each well of a 24 well plate. The cell number to be seeded was selected based on that investigated in a previous study (Goldbaum, 2019). The volume in each well was filled up

to 500 µL per well with DMEM + 5 % FCS. No antibiotic was added in the process of seeding. 24 well plates were incubated overnight at 37°C, 5 % CO_2.

b) Primary human keratinocytes: Primary human keratinocytes were used as the mammalian cell material for testing the endocytosis assay. Cells were carefully detached from the flasks using 1 mL, 0.05 % (v/v) trypsin solution. Cells were centrifuged at 900 rpm for 5 minutes and supernatant was discarded. Pellets were resuspended in KGM2 without supplements and cell density was determined by flow cytometry (see section 3.2, 3.3 and 3.7). Approximately 110,000 cells/cm^2 were seeded on a coverslip in each well of a 24 well plate and the volume was filled up to 500 µL per well with KGM2 containing nutrients. No antibiotic was added in the process of seeding. 24 well plates were incubated overnight at 37°C, 5 % CO_2.

ii) Keratinocyte starvation:

A previous study demonstrated that starvation conditions resulted in enhanced PAR2 density on the cell surface of keratinocytes relative to standard culturing conditions (Goldbaum, 2019). Hence the keratinocyte-starvation step was included in the endocytosis assay protocol.

a) HaCaT cells: DMEM + 5 % FCS was removed from the previously seeded HaCaT cells and they were thoroughly washed thrice by pipetting 500 µL prewarmed PBS per well followed by gently rinsing and discarding the liquid. 500 µL DMEM without FCS was added per well of a 24 well plate. No antibiotic was used in the process of cell starvation. Plates were further incubated overnight at 37°C, 5 % CO_2.

b) Primary human keratinocytes:

KGM2 was removed from the primary keratinocytes and they were very carefully washed thrice by pipetting 500 µL prewarmed PBS per well followed by gently rinsing and discarding the liquid. 500 µL KGM2 without nutrients were added per well of a 24 well plate. No antibiotics were used in the process of cell starvation. Plates were incubated for 8 hours at 37°C, 5% CO_2.

iii) Incubation of cyanobacteria with keratinocytes:

Cyanobacteria PCC 7942 were aliquoted under sterile conditions and P8 was covalently conjugated to the surface molecules of cyanobacteria PCC 7942 as described under section 3.5. Coverslips from 24 well plates with attached HaCaT cells or primary human keratinocytes were

transferred to wells of a new 24 well plate to eliminate the cells attached to the bottom of the wells but not the coverslip. 500 µL fresh DMEM without FCS and 500 µL KGM2 without nutrients were added per well of a 24 well plate for HaCaT cells and primary human keratinocytes, respectively. 1 million P8-conjugated cyanobacteria PCC 7942 resuspended in PBS were added to the keratinocytes in their respective media in 24 well plates at a ratio of at least 1:10 of keratinocytes to cyanobacteria PCC 7942, respectively. Various control samples were set up as described under Methods. The plates were incubated for up to 24 hours at 30°C in the dark to slow down proliferation processes of cyanobacteria PCC 7942.

iv) Immunocytochemical staining:

Incubations of cyanobacteria PCC 7942 and HaCaT cells or primary human keratinocytes were stopped at various time points by removing the incubation media and subsequent washing each well thoroughly, thrice with 500 µL PBS. Cells were fixed under a fume hood, with 300 µL, of a 4 % (v/v) paraformaldehyde (PFA) containing PBS solution for 10 minutes at room temperature. 4 % (v/v) PFA solution was carefully removed and discarded appropriately. Each well was again washed once with 500 µL PBS. Cell permeabilisation was achieved by addition of 200 µL ice-cold methanol to the cells under a fume hood and subsequent incubation for 2 minutes at room temperature. Methanol was removed and cells were washed thrice with 500 µL PBS containing 3 % (v/v) goat serum. Primary antibody binding to a cellular protein of keratinocytes (β-actin, Cytokeratin-14, PAR2) were diluted in PBS + 3 % (v/v) goat serum. 300 µL primary antibody solution was added to the fixed and permeabilized cells and cells were incubated for 2 hours on a shaker at room temperature. After 2 hours, antibody solution were removed and cells were washed thrice with 500 µL PBS containing 3 % (v/v) goat serum. Fluorophore-coupled secondary antibody (Indodicarbocyanine (Cy5) or Indocarbocyanine (Cy3) or Cyanine (Cy2)) was diluted in PBS containing 3 % (v/v) goat serum. Cells were incubated with 300 µL secondary antibody solution for 2 hours on a shaker in the dark at room temperature. Secondary antibody solution was removed and cells were washed thrice with PBS + 3 % (v/v) goat serum in order to remove unbound antibodies. 8 µL Vectashield mounting solution (containing the nuclear stain DAPI) per coverslip was pipetted onto a glass slide. Coverslips with antibody-treated keratinocytes were flipped onto the mounting solution with cells facing the glass slide. Slides were stored at 4°C or analysed by fluorescence microscopy (Refer section 3.8).

3.7 Flow cytometry

Flow cytometry/Fluorescence-activated cell sorting (FACS) is a technique used to detect and measure various physicochemical characteristics of cells or particles (Picot, *et. al.*, 2012). Flow cytometry was used in this work to determine the cyanobacteria PCC 7942 cell count and viability by analysing the fluorescence generated by their major light-absorbing chromophores termed phycocyanobilins which covalently bind to the accessory photosynthetic pigments phycobiliproteins (Pheiss & Kosuge, 1970). This technique was also used to analyse the efficiency of P8-conjugation to cyanobacteria PCC 7942 surface molecules by measuring the fluorescence of fluorescein 5-FAM as part of P8 itself. Cell count of HaCaT cells and primary keratinocytes was also determined by flow cytometry. Measuring forward scatter (FSC) and side scatter (SSC) of cells provide characteristic information on cell size, shape and granularity. Data was collected by CyFlow Cube 16 software and analysed using FCS Express 7.

3.8 Fluorescence microscopy

3.8.1 Epifluorescence microscopy

Immunochemically stained keratinocyte samples were analysed by epi-fluorescence microscopy using common fluorescence filter sets for DAPI, FITC and TRITC. Fluorescence microscopy images were captured using Axioplan 2 Plus Microscope and processed through the Case Data Manager software. Image analysis was performed using InkScape.

3.8.2 High resolution confocal laser scanning microscopy (CLSM)

Confocal Laser Scanning Microscopy (CLSM) is an optical imaging technique that increases the optical resolution and contrast by eliminating scattered light signals and also enables analysis of micrographs at different depths of the sample (Pawley, 2006). CLSM was performed in order to investigate interactions and localisation of cyanobacteria PCC 7942 with HaCaT cells and primary human keratinocytes. Zeiss LSM 880 with Airyscan having one major beam splitter for four laser lines: Argon laser (458, 488 and 514 nm), HeNe-laser (543 nm), DPSS-laser (561 nm) HeNe-laser (633 nm) was used. Samples were analysed at 20x magnification using fluorescence filter sets for DAPI, FITC, TRITC and Cy5. Zeiss Zen black software was used to capture and analyse images.

3.9. Appendix

3.9.1 Materials

Table 3.1. Composition of salt solution 1 (anhydrous salts) for the ESAW medium (Berges *et. al.*, 2001; Harrison *et. al.*, 1980).

Component	Quantity (g L^{-1})	Final concentration (M)	Supplier II
NaCl	21.194	$3.63 \cdot 10^{-1}$	VWR International (Radnor, PE, USA)
Na_2SO_4	3.550	$2.50 \cdot 10^{-2}$	Sigma-Aldrich (St. Louis, MO, USA)
KCl	0.599	$8.03 \cdot 10^{-3}$	Janssen-Cilag (Neuss, Germany)
$NaHCO_3$	0.174	$2.07 \cdot 10^{-3}$	Sigma-Aldrich
KBr	0.0863	$7.25 \cdot 10^{-4}$	Acros Organics, Thermo Fisher Scientific
NaF	0.0028	$6.67 \cdot 10^{-5}$	Sigma-Aldrich

Table 3.2. Composition of the salt solution 2 (hydrated salts) for the ESAW medium (Berges *et. al.*, 2001; Harrison *et. al.*, 1980).

Component	Quantity (g L^{-1})	Final concentration (M)	Supplier II
$MgCl_2 \cdot 6\ H_2O$	9.592	$4.71 \cdot 10^{-2}$	Honeywell Fluka™, Thermo Fisher Scientific (Waltham, MA, USA)
$CaCl_2 \cdot 2\ H_2O$	1.344	$9.14 \cdot 10^{-3}$	Merck (Darmstadt, Germany)
$SrCl_2 \cdot 6\ H_2O$	0.0218	$8.18 \cdot 10^{-5}$	Acros Organics, Thermo Fisher Scientific

Table 3.3. Stock solutions for major nutrients for the ESAW medium (Berges *et. al.*, 2001; Harrison *et. al.*, 1980). The dry ingredients were dissolved in Milli-Q water, sterile filtered (0.22 µm) and stored at 4°C. 1 or 2 mL of the major nutrients was added individually per litre of ESAW Medium.

Component	Stock concentration (g L^{-1})	Quantity (ml L^{-1})	Final concentration (M)	Supplier
$NaNO_3$	46.67	1	$5.49 \cdot 10^{-4}$	Merck (Darmstadt, Germany)
$NaH_2PO_4 \cdot H_2O$	3.094	1	$2.24 \cdot 10^{-5}$	Honeywell Fluka, Thermo Fisher Scientific (Waltham, MA, USA)

$Na_2SiO_3 \cdot 9\ H_2O$	15	2	$1.06 \cdot 10^{-4}$	Acros Organics, Thermo Fisher Scientific

Table 3.4. Stock solutions for the iron-EDTA stock solution for the ESAW medium (Berges et. al., 2001; Harrison et. al., 1980). The dry ingredients were dissolved individually in Milli-Q water and sterile filtered (0.22 µm) prior to storage at 4°C. 1 mL of iron-EDTA stock solution was added per litre of ESAW medium.

Component	Stock concentration	Quantity ($g\ L^{-1}$)	Final concentration (M)	Supplier II
$Na_2EDTA \cdot 2\ H_2O$	-	2.44	$6.56 \cdot 10^{-6}$	Carl Roth (Karlsruhe, Germany)
$FeCl_3 \cdot 6\ H_2O$	-	1.77	$6.55 \cdot 10^{-6}$	Merck (Darmstadt, Germany)

Table 3.5. Stock solutions for the trace metal stock solution for the ESAW medium (Berges et. al., 2001; Harrison et. al., 1980). The dry ingredients were dissolved individually in Milli-Q water and sterile filtered (0.22 µm) prior to storage at 4°C. 1 mL of trace metal stock solution was added per litre of ESAW medium.

Component	Stock concentration	Quantity	Final concentration (M)	Supplier II
$Na_2EDTA \cdot 2\ H_2O$	-	$3.09\ g\ L^{-1}$	$8.30 \cdot 10^{-6}$	PanReac AppliChem, ITW Reagents
$ZnSO_4 \cdot 7\ H_2O$	-	$0.073\ g\ L^{-1}$	$2.54 \cdot 10^{-7}$	Merck (Darmstadt, Germany)
$CoSO_4 \cdot 7\ H_2O$	-	$0.016\ g\ L^{-1}$	$5.69 \cdot 10^{-8}$	Merck
$MnSO_4 \cdot 4\ H_2O$	-	$0.54\ g\ L^{-1}$	$2.42 \cdot 10^{-6}$	Merck
$Na_2MoO_4 \cdot 2\ H_2O$[g]	$1.48\ g\ L^{-1}$	$1\ mL\ L^{-1}$	$6.12 \cdot 10^{-9}$	Merck
Na_2SeO_3[g]	$0.173\ g\ L^{-1}$	$1\ mL\ L^{-1}$	$1.00 \cdot 10^{-9}$	Sigma-Aldrich
$NiCl_2 \cdot 6\ H_2O$[g]	$1.49\ g\ L^{-1}$	$1\ mL\ L^{-1}$	$6.27 \cdot 10^{-9}$	Sigma-Aldrich

Table 3.6. Stock solutions for the vitamin stock solution for the ESAW medium (Berges et. al., 2001; Harrison et. al., 1980). The dry ingredients were dissolved individually in Milli-Q water. The dry ingredients were dissolved individually in Milli-Q water and sterile filtered (0.22 µm) prior to storage at 4°C. 1 mL of the vitamin solution was added per litre of ESAW medium.

Component	Stock concentration	Quantity	Final concentration (M)	Supplier

Thiamine · HCl (vit. B_1)	-	0.1 g L^{-1}	$2.96 \cdot 10^{-7}$	Sigma-Aldrich (St. Louis, MO, USA)
Biotin (vit. H)	1.0 g L^{-1}	1 mL L^{-1}	$4.09 \cdot 10^{-9}$	Sigma-Aldrich
Cyanocobalamin (vit. B_{12})	2.0 g L^{-1}	1 mL L^{-1}	$1.48 \cdot 10^{-9}$	Merck (Darmstadt, Germany)

Example 1 Selection of a cyanobacteria strain suitable for the generation of neo-endosymbiotic keratinocytes

Adaptation potential – The selection of a suitable and competent cyanobacteria strain for optimal light-controlled oxygen production after uptake into human primary keratinocytes has been investigated first as an essential factor for their intended applications.

For example, a suitable cyanobacteria strain has to be biocompatible with human keratinocytes regarding potential cytotoxicity. In addition, the light induced oxygen production should be effective under soft UV radiation conditions to prevent possible skin cell damage in medical applications. Therefore, several cyanobacteria strains of the genus *Synechococcus* have been tested regarding the characteristics mentioned before (Fig 10). In addition, one significant and essential advantage of *Synechococcus* represents the ability to adapt its metabolism to higher environmental temperatures, up to 30°C as well as to different pH and salt concentrations. Along this line, we have determined growth curves of the sweet water cyanobacteria strain PCC7942 under different conditions (Fig. 11).

The strain PCC7942 showed relatively high tolerance to applied culturing conditions such as 30°C, pH 7.42 and 30% salinity - relative to that of ESAW (Enriched seawater artificial water) 10 - medium, together mimicking cytosolic conditions within human keratinocytes. It should be noted that the overall average temperature of the upper skin (epidermis, containing keratinocytes) layer is around 26°C. Thus, this temperature adaptation process depicts a fundamental aspect of the applicability of the strain of Cyanobacteria for medicinal applications as aspired.

Cytotoxicity – Another important experimental parameter being essential for the success of the methods of the present invention represents the determination of potential cytotoxic effects of cyanobacteria on isolated primary keratinocytes. To investigate the cytotoxic potential, cell culture experiments were conducted using co-cultures of cyanobacteria (Synechococcus sp.) and human keratinocytes and determined cell viability using different densities of

Synechococcus sp. for 72 h of co-culture. Results did not show any evidence of dose dependent cytotoxic effects when co-cultivating keratinocytes together with cyanobacteria (Fig. 12). Along with our findings, Cohen *et. al.* have recently reported the successful co-cultivation of cyanobacteria together with heart muscle cells (cardiomyocytes) in a similar set-up, without any evidence of cytotoxic effects 11. In addition, during cultivation of human primary keratinocytes in the presence of cyanobacteria cell lysate no cytotoxicity or other effects on keratinocyte viability or proliferation were observed. In summary, these experimental results clearly demonstrate the general biocompatibility of both cell types when co-cultured and thus represent the fundamental basis for the successful combination of human or porcine keratinocytes and cyanobacteria in order to generate neo-endosymbiotic keratinocytes.

Example 2 Immunological characterization of isolated primary human keratinocytes

Section 4.1

Primary human keratinocytes were isolated and characterised as described below. A protocol for the isolation and cultivation of primary keratinocytes from human skin tissue samples has been established and optimised. In this study, primary keratinocytes were isolated from human foreskin samples from children between 1 to 4 years of age (Example 4, Section 4.3.1). To identify the cell type which was isolated from human skin tissue, immunocytochemical (ICC) staining was performed by making use of cell type specific markers as described under methods (Section Endocytosis assay). Antibodies against cytokeratin 14 (CK-14, a basal keratinocyte marker) (Harnden & Southgate, 1997) and PAR2 were used to selectively stain the components. Secondary antibodies either coupled to indocarbocyanine (Cy3) or fluorescein isothiocyanate (FITC) were used to detect PAR2 and CK-14, respectively. DAPI stain was used to designate the nuclear locations of isolated cells.

Keratinocytes isolated from human foreskin tissue samples were successfully isolated and cultured (Figure 5 A). 5-10% fibroblasts per flask were observed along with keratinocytes in the cultures and showed only nuclei and PAR2 staining without cytoskeletal staining due to use of the keratinocyte specific cell marker CK-14 (Fig. 5). Results indicated the localization of PAR2 (red) around nuclei (blue) of the cultured primary keratinocytes (Figure 5 A-C). Negative control where the primary keratinocytes were incubated with Cy3 and fluorescein fluorophore-coupled secondary antibodies without addition of respective primary antibody did not show any immunological staining (Figure 5 E-H). This illustrated the accuracy of detected elements and target specificity of the secondary antibody towards primary antibody used in

ICC. These results demonstrated the successful isolation and cultivation of primary keratinocytes from human foreskin tissue samples. The results are prominent, since the isolation and cultivation of primary cells often faces issues including the occurrence of many other cell types in the culture, lower rate of cell proliferation, higher chances of contamination which might come from the tissue itself. The cultivated primary human keratinocytes were further investigated for their interactions with the biochemically modified cyanobacteria.

Example 3 Survival of cyanobacteria *S. elongatus* PCC 7942 under cytosolic like conditions

Section 4.2

Previously as a part of the 'Endosymbiont' project, it has been demonstrated that the cyanobacteria strain *S. elongatus* PCC 7942 remains viable under cultivation conditions mimicking those found in keratinocyte cytosol. For example, *S. elongatus* PCC 7942 tolerate cytosol-like conditions including 30°C temperature, 7.4 pH and reduced salinity relative to their natural habitat conditions. However, these simplified experiments in principle cover physiological parameters such as pH, temperature and salt content but lack to mimic the presence of cytosolic biomolecules such organic ions, proteins and small vesicles. Hence, one central objective was to study the survival of *S. elongatus* PCC7942 in the presence of cytosol extracts isolated from HaCaT cells (human keratinocyte cell line) (Holden & Horton, 2009).

The cytosol was extracted from cultured HaCaT cells. In brief, HaCaT cells were detached from cell culture tissue plates, counted using flow cytometry (Section 3.6), mechanically broken down, and cell lysate was centrifuged to separate cell debris from HaCaT cytosol as described in section 3.4. Before utilizing the cytosol, cell lysate was analysed for confirming cell lysis.

The measurement of forward scatter (FSC) that indicates cellular shape and size, and side scatter (SSC) that indicates cellular granularity allowed allocation of the population representing viable HaCaT cells marked with the green region 'Viable HaCaT' (Fig. 6 A-C). Complete cell lysis was avoided to prevent destruction of cytoplasmic organelles *e.g.* such as lysosomes, which may also play an essential role in the survival of PCC7942 within the cytosol and would otherwise be missing when mimicking cytosol conditions. Approximately 3 million cells were measured by flow cytometry (Section 3.6), aliquoted and mechanically lysed (Fig.

6 B) but only one third portion of the viable cells was diminished (Fig. 6 C). Hence, the final cytosol was assumed to have been extracted from 1 million instead of 3 million HaCaT cells.

In order to exchange the cyanobacteria culture medium (30% osmolarity of ESAW) to PBS, a suspension containing approximately 1 million cells of *S. elongatus* PCC 7942 was centrifuged at 5000 rpm for 10 minutes at room temperature 30% ESAW and the cell pellet was resuspended in PBS. Subsequently, the cyanobacteria PCC 7942 in PBS were inoculated in cytosol extracts isolated from approximately 1 million HaCaT cells. Incubations were carried out for up to 48 hours in a phytotron at 30°C with 12 hours light and dark phases.

Fluorescence signals of phycocyanobilin (PCB) pigment of viable *S. elongatus* PCC 7942 incubated in cytosol extracts (Figure 7, black curve, mainly the upper curve) clearly matches that of *S. elongatus* PCC 7942 incubated in 30% ESAW medium (Figure 7, red curve). These results indicate that there are no significant differences in the viability of *S. elongatus* PCC 7942 when incubated in cytosol extracts relative to cyanobacteria incubated in 30% ESAW medium. The curves of both, sample and control conditions shift from lower to higher PCB concentrations, indicated by the increase in relative fluorescence intensity per cell as the time progresses from 3 to 48 hours (Figure 7 A-C). This might suggest the cyanobacteria being in the process of adaptation as a consequence of the changing conditions, which seemed to resemble in both sample and control conditions (Figure 7 A-C). These preliminary results provide good evidence that *S. elongatus* PCC 7942 will remain functional in the cytosol of living keratinocytes after cellular uptake as hypothesised in this study.

Example 4 Biochemical modifications of cell surface molecules of cyanobacteria *S. elongatus* PCC 7942

Section 4.3

During the experiments performed in connection with the present invention, *S. elongatus* PCC 7942 strain was identified as a promising candidate for being functional in cytosol of human keratinocytes and consequently for endosymbiont formation. In addition, the potential of chemical conjugation of the previously generated PAR2 agonist P8 molecule to surface molecules of cyanobacteria PCC 7942 was investigated. Such P8-conjugated cyanobacteria PCC 7942 have the potential to be endocytosed as it has been demonstrated for P8-decorated nanoparticles (WO 2019/057988 A1). Surface modifications were attempted to generate amide bonds between the amino group of the lysine linker of P8 and carboxyl groups on the surface

molecules of *S. elongatus* PCC 7942. Since an amide bond does not spontaneously form at physiological conditions, coupling reagents EDC (N′-ethylcarbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) were used for cross-linking purpose. EDC activates carboxyl groups and forms unstable O-acylisourea intermediate ester which spontaneously react with primary amines to form amide bonds. Addition of NHS to the reaction mixture results in the more stable NHS-ester derivate, which increases efficiency of amide bond formation.

The outer membrane constituents of cyanobacteria include glycoproteins as well as the acidic sugars, glucuronic and galacturonic acid (Kehr & Dittmann, 2015) that have free carboxyl groups which should be target structures for the amidation reaction with EDC and NHS. In this section, the possibility of amine reactive intermediate formation on the surface of cyanobacteria was tested before providing primary amine group at the lysine linker subunit of P8. Optimisations were made considering various reaction parameters including reaction buffer, temperature, reagent concentrations to carry out such a reaction.

4.3.1. Effects of coupling reagents on viability of S. elongatus PCC 7942

A reaction mixture was prepared in a 15 mL Falcon tube by aliquoting approximately 1 million cells of cyanobacteria *S. elongatus* PCC 7942 counted by flow cytometry (Section 3.7). Coupling reagents 10 µM N′-ethylcarbodiimide hydrochloride (EDC) and 20 µM N-hydroxysuccinimide (NHS) were added at their final concentrations from 4 mM stock solutions. PBS was added to make up the volume to 1 mL and the reaction mixture was incubated on a rotation wheel, in the dark to prevent cell proliferation of cyanobacteria, at room temperature for 1 hour. The fluorescence of phycocyanobilin (PCB) and allophycocyanin (APC) which are photosynthetic pigments of cyanobacteria were measured in flow cytometer after excitation at 638 nm, to analyse any adverse effects of the coupling reaction conditions on viability of cyanobacteria cell populations.

The gated region ‘PCC 7942’ (Fig. 8 A-C) was marked using a control condition where *S. elongatus* PCC 7942 aliquoted from their *in vitro* culture conditions and measured at given wavelengths (Fig. 8 B). The background signals originated from PBS were omitted from the gated region (Fig. 8 A). Hence, the gated region shows presence of viable *S. elongatus* PCC 7942. This analysis was performed one single time, where the graph showed insignificant variation of photosynthetic pigment densities per cell of cyanobacteria incubated with EDC

and NHS (Fig. 8 C) as compared to the cells incubated without any coupling reagents (Fig. 8 B). Perhaps, it can be observed that the incubation with coupling reagents EDC and NHS at given concentrations can be tolerated for up to 1 hour without inducing significant compromise of *S. elongatus* PCC 7942 cell viability.

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4.3.2. Characterisation of the coupling reaction efficiency

After characterising the survival of cyanobacteria in the presence of coupling reagents (Figure 5. A-C), the protocol for chemical modification of surface molecules of *S. elongatus* PCC 7942 was modified further. The potential NHS ester intermediates formed on the cyanobacterial 10 surface using EDC and NHS allow efficient conjugation to primary amines which might also be present on the same or neighbouring *S. elongatus* PCC 7942 cells. Such an intercellular crosslinking may form clumps of cells that can be detected by flow cytometry analysis. Addition of neutral amino acid residues can provide free primary amines to react with remaining NHS esters and inactivate them while preventing conjugation between the *S.* 15 *elongatus* PCC 7942 cells. To verify these factors, glycine was added at a final concentration of 1 mM after completion of 1-hour incubation of *S. elongatus* PCC 7942 with the coupling reagents (Section 4.3.1). The incubations were centrifuged at 5000 rpm, for 10 minutes at room temperature to pellet the cyanobacteria. Two different concentrations 4 µM and 20 µM of both coupling reagents EDC and NHS were used. Samples were analysed by flow cytometry for 20 forward scatter and phycocyanobilin pigment concentrations per cell.

Table 4.1: Composition of incubations to study the EDC-NHS induced crosslinking reaction.

Dot-plot description in Fig. 9	Components of the protocol			
	Cyanobacteria	EDC	NHS	Glycine
Green	+	-	-	-
Red	+	+	+	-
Blue	+	+	+	+

Forward scatter (FSC) plotted against relative fluorescence intensity measured at Em638 nm 25 within the circular region indicate the population of *S. elongatus* PCC 7942. Use of crosslinking reagents at both higher (20 µM) and five-fold lower (4 µM) concentrations seemed to have negligible adverse effects on the vitality cyanobacterial populations (Fig. 9 A-B). Cells appeared to tolerate the presence of 1 mM glycine for up to 15 minutes and underwent very small decrease in FSC value indicating slight detectable reduction in shape relative to the 30 sample without glycine treatment (Fig. 9). A subsequent centrifugation step and pellet

resuspension in PBS for the removal of excess coupling reagents or reaction by-products or glycine didn't generate detectable morphological changes or cyanobacterial cell-clumps, indicated by the FSC value in any of the samples and controls. This characterization step was utilized to optimize the cyanobacterial chemical surface coupling protocol. The efficiency of the protocol and fate of the experiment was studied further by addition of P8 molecule to the reaction mixture that could be detected by flow cytometry.

4.4 Protease activated receptor 2 (PAR2) agonist P8 to surface molecules of *S. elongatus* PCC 7942

The effects of coupling reagents EDC and NHS were determined before on survival and morphological characteristics of the *S. elongatus* PCC 7942 strain (Section 4.3). Finally, experiments were conducted using EDC and NHS to chemically conjugate carboxyl groups of surface molecules on cyanobacterial outer membrane to the primary amine group of the lysine residue being part of the PAR2 agonist P8. The experiment aimed at a successful conjugation of cyanobacteria *S. elongatus* PCC 7942 with P8 molecules through formation of amide bonds. The specially designed P8 molecule having PAR2 agonist hexapeptide which was hypothesized to play a role in developing interactions of cyanobacteria *S. elongatus* PCC 7942 with human keratinocytes was also evaluated here.

4.4.1 Concentration determination of P8 in methanol

To quantify P8 molecules present in the stock solution by performing a fluorescence dependent quantification first a calibration curve was generated. The amount of P8 molecules present in the stock solution were determined by measuring fluorescence of FAM-5 (5-carboxy-fluorescein). The fluorescence measurements for calibration were determined using Fmoc-Lys(5-Fam) [N^{α}-(9-fluorenylmethoxycarbonyl)-N^{ε}-(5-carboxy-fluorescein)-L-lysine] as reference, since it is a building block of P8. Measurements were performed using a microtitre plate photometer.

A linear correlation between the relative fluorescence intensity and concentration of Fmoc-Lys(5-Fam) is necessary to determine the amount of P8 in samples of unknown concentration. Fluorescence intensities for both standard and P8 were measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Unlike P8, Fmoc-Lys(5-Fam) has very poor water-solubility. Therefore, the fluorescence intensities of Fmoc-Lys(5-Fam) and the P8-

samples of unknown concentration were measured over a range of concentrations in methanol, although the final P8 solution was prepared in milli-Q to further crosslink with surface molecules of *S. elongatus* PCC 7942.

The measurement has shown a linear correlation between the fluorescence intensity and the Fmoc-Lys(5-Fam) concentration between 1 mM and 2 mM. At higher concentrations, quenching effects were observed (Fig 9 C). Since a linear correlation between the concentration and fluorescence intensity was observed, this method could be used to determine the amount of P8 present in the stock solution which was used in further experiments.

4.4.2 Chemical conjugation of P8 to surface molecules of S. elongatus PCC 7942

Determination of the amount of P8 in stock solution enabled the addition of known number of P8 molecules to the reaction mixture. P8 was further used to crosslink onto the surface of S. elongatus PCC 7942 by means of crosslinking reagents EDC and NHS. P8 molecule is designed to have a reactive primary amine that provides the ability for chemical conjugation with NHS-ester intermediates.

Previous optimizations in the protocol to generate NHS ester on cyanobacterial surface were performed using coupling reagents EDC and NHS. A reaction mixture was prepared in a Falcon tube by aliquoting approximately 1 million cells of *S. elongatus* PCC 7942 together with 10 µM EDC, 20 µM NHS and 1 µM P8. PBS was added to make up the volume to 1 mL and the reaction mixture was incubated at room temperature for 1 hour on a rotation wheel, in the dark to prevent cell proliferation of cyanobacteria. After 1 hour of incubation, glycine was added to yield a final concentration of 1 mM and reaction mix was further incubated for additional 15 minutes. Falcon tubes were centrifuged at 5000 rpm, for 10 minutes at room temperature to facilitate closer interactions of cells and other components within the reaction mixture. This step can lead to inactivate the unreacted NHS-ester intermediates with primary amine from either P8 or glycine. Cyanobacteria pellets were resuspended in PBS to remove the unreacted components and reaction by-products remained in the supernatant after centrifugation. Flow cytometry was performed to detect the association of P8 with cyanobacteria *S. elongatus* PCC 7942.

The fluorescence measurements plotted for fluorescein FAM-5 (FL2) against phycocyanobilin (FL5) may correspond to *S. elongatus* PCC 7942 populations which are likely to be viable due to the relatively high PCB signals (FL5) associated to intact pigment structures. Flow cytometry analysis of the reaction mixture revealed two distinct populations after plotting the relative fluorescence intensity of fluorescein FAM-5 (FL2) against that of phycocyanobilin (FL5) correlating cyanobacteria as seen in Fig. 13 A. Comparing the resulting dot plot **A** to those of samples only containing cyanobacteria (**B)** or PBS (**C)** clearly indicates that the major population observed is positive for both FAM-5 and PCB suggesting an association of P8 with cyanobacteria (Fig. 13 A). The minor group of cells that appeared in the gated region (Fig. 13 A) with low FAM-5 and high PCB values might be a population of cyanobacteria not associated with P8. The signals with low FAM-5 and PCB values located outside of the gated regions may originate from PBS used as a solvent. Results from this experiment demonstrated the association of P8 to cyanobacteria but didn't give a clear idea of their linking pattern. However, this experiment indicated that the reaction conditions will not have any severe cytotoxic effects on cyanobacteria *S. elongatus* PCC 7942 as well.

4.4.3 Interaction study of HaCaT cells with P8-conjugated cyanobacteria S. elongatus PCC 7942

P8 binding and activation of PAR-expressing HaCaT cells was tested by establishing an 'endocytosis assay' of P8-modified cyanobacteria with HaCaT cells. The hexapeptide part of P8 was hypothesized to bind to cell surface PAR2 on HaCaT cells. Cyanobacteria *S. elongatus* PCC 7942 were surface modified by chemical attachment of P8 using the above-mentioned protocol (Section 4.4.2). Approximately 90,000 HaCaT cells/ cm^2 were seeded in each well of a coverslip containing 24-well plate and incubated in 500 µL DMEM containing 5 % (w/v) FCS overnight at 37°C, 5 % CO_2 (Section 3.6). A previous study has demonstrated that starvation conditions result in enhanced PAR2 expression on the surface of keratinocytes. Therefore, the medium was replaced after previous incubation with 500 µL DMEM without FCS in each well and HaCaT cells were starved overnight at 37°C, 5 % CO_2.

Endocytosis assay was performed in duplicates on serum starved HaCaT cells by addition of P8-conjugated cyanobacteria PCC 7942 in 500 µL DMEM without FCS in each well of a 24 well plate (Section 3.6). Approximately 1 million P8-conjugated cyanobacteria PCC 7942 resuspended in PBS were added to the 90,000/cm^2 HaCaT cells in a ratio of 10:1 (10

cyanobacteria PCC 7942 per HaCaT cell). The plates were incubated for up to 15 hours at 30°C in the dark to slow down proliferation processes of cyanobacteria. Incubations were stopped at time points of 3, 6, 9 and 15 hours by removing the incubation medium and subsequent washing the cells. 500 µL of PBS was added to the cells, gently rinsed (by shaking) and discarded 3 times to remove non-associated cyanobacteria. After washing, cells were fixed and permeabilised as described under Methods (Section 3.6). Vectashield mounting solution, containing DAPI (DNA-binding fluorescent stain) was used for nuclei staining of HaCaT cells. Samples were analysed using common fluorescence filter sets for DAPI and Cy3 and an epifluorescence microscope. HaCaT cells incubated in the presence or absence of unmodified or modified cyanobacteria were used as controls.

Table 4.2: Composition of incubations to study the time dependent interactions of P8-conjugated cyanobacteria with HaCaT cells.

Image description	Assay components		
Fig. 14	HaCaT cells	P8	Cyanobacteria
A-D	+	-	-
A1-D1	+	-	+
A2-D2	+	+	+

Samples of the endocytosis assay showed a successful nuclear staining of HaCaT cells (Ex365 nm/Em420, blue signals) and detection of the phycocyanobilin pigment from cyanobacteria PCC 7942 (Ex546 nm/Em590 nm, red signals). The P8-associated cyanobacteria PCC 7942 clearly showed enhanced interactions with the HaCaT cells (Fig. 14 A2-D2) as compared to the HaCaT samples incubated in the presence of unmodified cyanobacteria PCC 7942 (Fig. 14 A1-D1), which clearly demonstrate P8 involvement. P8-associated cyanobacteria PCC 7942 incubated for 9 hours interacted with the HaCaT cells in a significantly higher number (Fig. 14 C2). This number was notably low for the same sample preparation incubated for 6 hours (Fig. 14 B2) and didn't seem to increase much as the time progressed towards 15 hours (Fig. 14 C2). This might suggest that the interactions between the P8-conjugated cyanobacteria PCC 7942 and HaCaT cells were established between time points of 6 and 9 hours. This experiment supported the previous result (Section 4.4.2) stating an association of P8 with the cyanobacteria *S. elongatus* PCC 7942.

A preliminary evaluation of the uptake efficiency in the experiment, with the incubation time of 9 hours, indicated that the efficiency is much higher than 5% as shown for other methods, e.g. in Agapakis at al. 2011. In particular, in a first count approach, the proportion of

cyanobacteria taken up in total by the HaCaT cells to the number of HaCaT cells as seen in Figs. 3 was estimated to about 40%.

Example 5 Elucidation of P8-attachement to *S. elongatus* PCC 7942

Section 4.5

4.5.1. Analysis of degree of P8-conjugation to cyanobacteria S. elongatus PCC 7942

Clear interactions of P8-conjugated cyanobacteria with HaCaT cells were observed using the endocytosis assay (Section 4.4.3). Although it emphasised the importance of P8-conjugation to cyanobacteria PCC 7942, the extent of P8 covalently conjugated with cyanobacterial surface molecules remained unknown.

Approximately 1 million cells of cyanobacteria *S. elongatus* PCC 7942 were incubated in 1 mL PBS containing 1 mM P8 and in the presence or absence of 20 µM EDC and 10 µM NHS for 1 h, in the dark at room temperature. Glycine was added to the reaction mix to yield a final concentration of 1mM and samples were further incubated for 15 minutes. After incubation cell samples were directly analysed by flow cytometry or centrifuged at 5000 rpm for 10 minutes, followed by pellet-resuspension in PBS. Fluorescence signals were measured by flow cytometry before and after removal of excess and unbound P8 from the reaction mixture by centrifugation (further referred as a washing step). For data analysis cyanobacterial count was plotted against P8-fluorescence.

Table 4.3: Protocol details including the description of incubations used to study the conjugation of P8 to cyanobacteria PCC 7942.

Fig. 2 Curve description	Components and steps of the protocol				
	Cyanobacteria	EDC	NHS	P8	Washing step
Black	+	-	-	-	-
Blue	+	+	+	+	-
Green	+	-	-	+	-
Purple	+	+	+	+	+
Red	+	-	-	+	+

The incubations of *S. elongatus* PCC 7942 in 1 mL PBS containing 1 mM P8 molecules with (Fig. 2, blue curve) or without (Fig. 2, green curve) EDC and NHS showed a slight difference in the distribution of fluorescence intensities (Fig. 2). Flow cytometry analysis of these samples

after washing (Fig. 2, purple and red curves) appeared in the range between 10^2-10^3 relative fluorescence intensity. They also resembled the distribution pattern of relative fluorescence intensity (RFI) per event (cell) observed before washing (blue and green curves). The slight differences in distribution of fluorescence intensities now appeared at a range of lower RFI (Fig. 2). This might be an effect of elimination of the excess P8 molecules which were non-covalently bound to the cyanobacteria. The washing process appeared to remove that portion of P8 molecules (Fig. 2). Perhaps, neither of the curves measured after washing steps overlapped fluorescent signals emerging from cyanobacteria without P8 (Fig. 2, black signal). This indicates incomplete elimination of P8 molecules after washing, both, in the presence and absence of coupling reagents (Fig. 2). This suggests another pattern of association other than conjugation to exist between P8 and cyanobacteria. This experiment signifies that the P8-conjugated cyanobacteria when incubated in the presence of EDC and NHS might have a combination of conjugated and loosely bound/adsorbed P8 onto their surfaces.

4.5.2. Effect of mode of P8-association in cyanobacteria-keratinocytes interactions

The cyanobacteria *S. elongatus* PCC 7942 incubated with EDC, NHS and P8 were assumed to have a combination of conjugated and adsorbed P8 onto their surfaces (Section 4.5.1). Hence, it was important to study whether and to which extend the proposed adsorbed fraction of P8 is involved in interaction with HaCaT cells as well as the potential role in triggering endocytosis.

P8-conjugated cyanobacteria were generated using the protocol described earlier (Section 4.4.2). 90,000/cm^2 HaCaT cells were seeded on each coverslip per well of a 24 well plate and serum starved overnight (Section 3.6). Starved HaCaT cells were used for the endocytosis assay as mentioned earlier (Section 4.4.3).

Endocytosis assay incubations were carried out for 15 hours at 30°C, in the dark. Samples were fixed, permeabilized and stained as described under Methods. Fluorescence signals were measured using an epifluorescence microscope. Following controls were set up with HaCaT cells for a detailed analysis: Addition of cyanobacteria incubated with P8 without coupling reagents EDC and NHS, cyanobacteria incubated with or without coupling reagents EDC and NHS, cyanobacteria with or without P8, and presence or absence of P8.

Table 4.4: Composition of incubations to study the interactions of P8-associated cyanobacteria with HaCaT cells for 15 hours.

Fig. 15 Image description	Assay components					
	HaCaT cells	Cyanobacteria	EDC	NHS	P8	Glycine
A	+	+	+	+	+	+
B	+	+	-	-	+	-
C	+	-	-	-	+	-
D	+	-	-	-	-	-
E	+	+	+	+	-	-
F	+	+	+	+	-	+
G	+	+	-	-	+	-
H	+	+	-	-	-	-

The endocytosis assay preparation showed a successful nuclear staining of HaCaT cells (blue signals) and detection of the phycocyanobilin pigment from cyanobacteria *S. elongatus* PCC 7942 (red signals). Although 90,000/cm^2 HaCaT cells were seeded on each coverslip, the cellular densities majorly varied throughout the area of coverslips (Fig. 15 A-H). Incubation of cyanobacteria PCC 7942 with soluble P8 in the absence of coupling reagents EDC and NHS lead to significantly less interactions with HaCaT cells (Fig. 15 B), compared to the same sample but incubated in the presence of coupling reagents (Fig. 15 A). This confirms the significant role of chemical conjugation of P8 with the surface molecules of cyanobacteria PCC 7942 leading to interactions with HaCaT cells. It also suggests that the presence of P8 along with the cyanobacteria PCC 7942 in a potentially loosely attached or in any other indeterminate form isn't sufficient to enhance such interactions with the HaCaT cells (Fig. 15 A, B, G). Populations of cyanobacteria PCC 7942 having activated NHS-ester intermediates incubated with HaCaT cells did not contribute to a significant addition of interactions as seen in the presence of P8 (Fig. 15 E). The HaCaT cells seemed to tolerate the presence of P8 molecules up to concentration of 1 µM for 15 hours (Fig. 15 C). This experiment signifies that the mode of P8-association to the surface of cyanobacteria PCC 7942 plays an essential role for enhancing their interactions with HaCaT cells (Fig. 15 A).

4.5.3 Characterization of P8 dissociation from the surface of S. elongatus PCC 7942

The experiments described herein provided strong evidence that the P8-association with cyanobacteria may be a combination of conjugated and adsorbed P8 when incubated in the presence of crosslinking reagents EDC and NHS. To understand and distinguish between covalently conjugated and potentially adsorbed P8 onto cyanobacterial surface molecules, an

experiment was conducted as follows. In the first step cyanobacteria were incubated with P8 in the presence and absence of EDC and NHS. In a second step both cyanobacteria preparations were centrifuged and resuspended in 1 ml of fresh PBS (Section 4.4.2). Second step was repeated 5 times and the supernatant from each washing was stored and fluorescence was subsequently measured at wavelength using a microtitre plate photometer. The cell pellets after fifth re-suspension were analyzed by flow cytometry.

Table 4.5: Protocol details including the description of incubations used to characterize P8 dissociation from cyanobacteria

Fig. 2 B, C - Curve description	Components and steps of the protocol				
	Cyanobacteria	EDC	NHS	P8	Washing step (5X)
Green	+	-	-	+	-
Red	+	-	-	+	+
Blue	+	+	+	+	+
Purple	+	-	-	-	+
Orange	-	-	-	-	-

Cyanobacteria PCC 7942 incubated in the presence (Fig. 2 B, C, blue curve) of EDC and NHS showed a significant reduction in relative fluorescence intensity of fluorescein FAM-5 relative to the incubations in the absence of coupling reagents (Fig. 2 B, C, green curve), which wasn't removed through 5-times washing steps. Cyanobacteria PCC 7942 incubated in the presence (Fig. 2 B, C, blue curve) of P8-EDC-NHS almost overlapped with signals from cyanobacteria PCC 7942 incubated in PBS without P8 (purple signals) (Fig. 2 B, C). This indicates that majority of the P8 was loosely bound to the cyanobacteria which dissociated from the cell surface by every washing step performed.

It was interesting to investigate whether the previously observed interactions of P8-conjugated cyanobacteria PCC 7942 with HaCaT cells might still be observable after dissociation of P8 from cyanobacteria surface by centrifugation and subsequent cell-pellet resuspension performed for 5 consecutive times. Endocytosis assay was performed as mentioned earlier (Section 4.4.3). 90,000/cm^2 HaCaT cells were seeded on each coverslip per well of a 24 well plate and serum starved overnight (Section 3.6). Starved HaCaT cells were used for the endocytosis assay and incubations were carried out for 15 hours at 30°C, in the dark. Fluorescent staining followed by epifluorescence microscopy was performed (Section 4.4.3).

Table 1.6: Components of incubations to study of effect of extensive washing of P8-conjugated cyanobacteria PCC 7942 on their interactions with HaCaT cells.

Fig. 16 Image description	Assay components					
	HaCaT cells	Cyanobacteria	EDC	NHS	P8	Glycine
A	+	+	+	+	+	+
B	+	+	-	-	+	-
C	+	-	-	-	-	-
D	+	+	-	-	-	-

The performed endocytosis assay using P8-conjugated cyanobacteria PCC 7942 preparations, which have been extensively washed before applying onto HaCaT cells showed clear nuclear staining (blue signals) for all samples as well as phycocyanobilin fluorescence for some sample (red signals). Although, 90,000/cm^2 HaCaT cells were seeded on each coverslip, the number of adhered cells appeared to be less (Fig. 16 A-D). This might be a consequence of cells being in unfavourable conditions, such as obtaining HaCaT cells from an already overgrown culture plate. The P8 chemically conjugated to the surface molecules of cyanobacteria PCC 7942 seemed to be inefficient in contributing to increased interactions with the HaCaT cells (Fig. 16 A). This might be a result of removal of P8 by means of many cycles of centrifugation and cell-pellet resuspension which might have removed even the covalently conjugated P8 along with adsorbed portion. Hence, other methods to selectively remove only adsorbed P8 molecules while keeping the outer membrane layer intact are needed for further conclusions.

Although, the determination of amount of covalently conjugated P8 with cyanobacteria PCC 7942 wasn't yet possible, these experiments provided evidence of potential time dependent dissociation of P8 molecules from cyanobacteria surface. To investigate this further, endocytosis assay conditions were used but in the absence of HaCaT cells. P8-conjugated cyanobacteria PCC 7942 were generated with addition of the coupling reagents EDC and NHS as described earlier (Section 4.4.2). Cyanobacteria PCC 7942 were further incubated in each well of a 4 well plate in 500 µL phenol red-free DMEM for 15 hours, in the dark at 30°C, and were analysed by flow cytometry.

Table 4.7: Protocol details including the description of incubations used to characterize time-dependent P8 dissociation from cyanobacteria

Fig. 2 D, E Curve description	Components and steps of the protocol					
	Cyanobacteria	EDC	NHS	P8	15 h incubation in DMEM	Washing step (1X)
Green	-	-	-	-	-	-
Red	+	+	+	+	-	+
Blue	+	+	+	+	+	-
Purple	+	+	+	+	+	+

The P8-conjugted cyanobacteria PCC 7942 incubated in DMEM for 15 h, in the dark at 30°C were measured before (Fig. 2 D, E, blue curve) and after (Fig. 2 D, E, purple curve) centrifugation at 5000 rpm, for 10 minutes at room temperature. Both these curves were very close to the background fluorescence range (Fig. 2 D, E), suggesting that a considerable amount of P8-dissociation occurs over the 15 hours incubation period of the endocytosis assay. This also suggests that even though an excess amount of P8 molecules associated with cyanobacteria PCC 7942 are incubated with HaCaT cells in the endocytosis assay, only the minor fraction of chemically conjugated P8 remains responsible for the enhanced interactions with the HaCaT cells.

P8-dissociation observed over 15 hours of incubation in DMEM was studied at smaller time points. Approximately 1 million P8-associated cyanobacteria were generated with addition of the coupling reagents EDC and NHS using a protocol prepared earlier (Section 4.4.2). The cyanobacteria PCC 7942 were further incubated in 500 µL phenol red-free DMEM for 6 hours, in the dark at 30°C. Incubations were stopped at every hour from 15 minutes to 6 hours and cells were analysed by flow cytometry in 1 mL phenol red-free DMEM without prior centrifugation. The sample along with various experimental conditions were measured as follows:

A) Sample condition where P8-conjugated cyanobacteria PCC 7942 were prepared by chemical conjugation using EDC and NHS, incubated for 1 h in PBS

B) Cyanobacteria PCC 7942 incubated with P8 for 15 minutes to bring their primary amines in the vicinity of cyanobacterial outer layer followed by an addition of EDC and NHS for 45 minutes, in PBS

C) Cyanobacteria PCC 7942 and P8 incubated without EDC and NHS in PBS to create cells having adsorbed P8 on their surfaces

D) Cyanobacteria PCC 7942 with 5 cycles of centrifugation and PBS-resuspension to remove presence of potential slime layer for more exposure of the outer membrane molecules, incubated with P8, EDC and NHS in PBS

E) Cyanobacteria PCC 7942 without any additives incubated for 1 h. in PBS (all concentrations as per section 4.4.2).

The sample condition where P8-conjugated cyanobacteria were prepared by chemical conjugation using EDC and NHS almost always showed fluorescence values higher than any other sample, yet the values were very close to the background of cyanobacteria PCC 7942 without P8 even when measured at 15 minutes (Fig. 17 A). No significant trend of increase or decrease of relative fluorescence of P8 was observed over time from 15 minutes to 6 hours (Fig. 17 A-D). Since the 0-hour measurement could not be determined due to technical difficulties, the majority of P8-dissociation from the surface of cyanobacteria PCC 7942 might already have taken place during the 15 minutes from start of incubation in DMEM (Fig. 17 A-D). The modifications made in generating chemically modified P8-associated cyanobacteria PCC 7942 (Fig. 17 B and D) didn't have any notable effect in increasing the conjugation between P8 and cyanobacteria PCC 7942. This experiment verified the stability, functionality and efficacy of the protocol designed during the thesis project for chemical attachment of P8 onto the surface molecules of cyanobacteria *S. elongatus* PCC 7942.

4.6 Investigation of cellular localization of cyanobacteria

The study of P8-conjugated cyanobacteria PCC 7942 and HaCaT cells demonstrated increased interactions between them when P8 molecule was chemically conjugated to surface molecules of the cyanobacteria PCC 7942 relative to untreated samples (Section 4.4 and 4.5). DAPI staining was used in combination with epifluorescence microscopy for estimating whether P8-conjugated cyanobacteria PCC 7942 are localized in close vicinity to the nucleus of HaCaT cells.

The optical filters of epifluorescence microscope could simultaneously detect only 3 fluorophores or fluorescent proteins without introducing a crosstalk or bleed-through artefacts due to significant spectral overlap. Hence, so far, nuclei of HaCaT cells (DAPI stain) and the

fluorescence of cyanobacterial phycocyanobilin (PCB stain) were imaged by epifluorescence microscopy. The P8 (fluorescein stain) fluorescence was not seen under the microscope, although it could be detected in flow cytometry (Ex488 nm). Hence, quadruple fluorescence staining was performed to additionally detect Cy3-immunocytostained cytokeratin-14 (CK-14) cytoskeletal protein from the keratinocytes to locate the cellular boundaries along with nuclei, P8, and phycocyanobilin fluorescence. Observations were made using a confocal laser scanning microscope (CLSM) which allowed quadruple fluorescence detection. CLSM could provide stronger fluorescence signals at higher intensity and allowed controlled image capture by scanning through the sample depths. Fluorescence emission spectra were detected at emission wavelengths of 457 nm, 517 nm, 566 nm, 656 nm. Corresponding laser lines were: 488 nm- the line of argon (Ar) laser, 405 nm- the line of diode UV laser, 543 nm and 633 nm- the line of helium-neon (HeNe) laser. Zeiss Zen black software was used to capture and analyze data.

4.6.1. Study of cellular localization of P8-conjugated cyanobacteria with HaCaT cells

Previously, the endocytosis assay of cyanobacteria PCC 7942 conjugated with P8 using the coupling reagents showed enhanced interactions with the HaCaT cells (Section 4.4.3). The experimental set-up was repeated on HaCaT cells and (Section 4.4.3 and 3.6) the quadruple fluorescence detection was examined using CLSM.

Table 4.8: Composition of incubation to study localization of P8-modified cyanobacteria with HaCaT cells

Fig. 18 Image description	Components of the assay					
	HaCaT cells	Cyanobacteria	EDC	NHS	P8	Glycine
A-D	+	+	+	+	+	+

The confocal laser scanning microscopy showed quadruple fluorescence staining by detecting Cy3-immunocytostained CK-14 intermediate filaments (Em566 nm, Cy3 channel) from the keratinocytes along with nuclei (Em457 nm, DAPI channel) and phycocyanobilin fluorescence (Em656 nm, Cy5 channel). Fluorescence signals of the CK-14 staining leaked heavily into the FITC channel (Em517 nm) (Figs. 18-20) due to remaining spectral bleed-through artefacts. CLSM analysis confirmed that the chemically P8-modified cyanobacteria PCC 7942 using

coupling reagents EDC and NHS show enhanced cellular localisation with the HaCaT cells (Fig. 18 A-D). CK-14 staining defined the HaCaT cell boundaries which contributed to an observation that most on the P8-conjugated cyanobacteria PCC 7942 interacting with the HaCaT cells were located within CK-14 stained regions of the cells (Fig. 18 A-D). A trend could be observed in most cases, where more than one P8-conjugated cyanobacteria were found localizing a single HaCaT cell (Fig. 18 B and D). Their cellular localisation could be investigated further using z-axis scanning provided by the CLSM that allowed controlled image capture through the sample depths (Fig. 19).

The HaCaT-cell samples incubated with P8-conjugated cyanobacteria PCC 7942 were vertically scanned from bottom layer to top layer to locate the occurrence of cyanobacteria. The vertical scanning was performed through 4 sample slices over a range of 6 µm (Fig. 19 C') and 6.9 µm (Fig. 19 D') for fluorescence microscopy images given above (Fig. 18 C and D, respectively).

In several cases, the Z-axis/vertically scanned CLSM images displayed the P8-conjugated cyanobacteria PCC 7942 and HaCaT cells to be located in the same focal plane. All three fluorophores labelling nuclei, CK-14 and phycocyanobilin respectively were focused in the same sample layer. This is first evidence of P8-conjugated cyanobacteria PCC 7942 present within the cytosol of cultured HaCaT cells.

Table 4.9*:* Composition of control-condition incubations to study localization of P8-conjugated cyanobacteria with HaCaT cells

Fig. 20 Image description	Components of the assay						
	HaCaT cells	Cyanobacteria	EDC	NHS	P8	Glycine	Primary antibody
A	+	+	-	-	+	+	+
B	+	+	+	+	-	-	+
C	+	+	+	+	-	+	+
D	+	+	-	-	-	-	+
E	+	-	-	-	-	-	+
F	+	-	-	-	+	-	+
G	+	-	-	-	-	-	-

None of the control conditions (Fig. 19) showed increase in the cyanobacteria-HaCaT cell interactions as seen in samples having P8 molecule chemically conjugated to the surface molecules of cyanobacteria *S. elongatus* PCC 7942. Although some samples showed very weak CK-14 staining or staining only around cell nuclei (Fig. 19 A-D, F). It is assumed that the sample preparations for control conditions were 5 days old when observed under CLSM which might have caused photobleaching.

Discussion

The preceding Examples describe experiments directed to chemical attachment of P8 to surface molecules of cyanobacteria *Synechococcus elongatus* PCC 7942 and characterisation of interactions between P8-conjugated cyanobacteria and human keratinocytes. Such P8-conjugated cyanobacteria were utilized to study their localization within HaCaT cells and subsequently within primary human keratinocytes.

The experiments involved isolation and culturing of primary human keratinocytes isolated from human foreskin tissue samples (Section 3.3.1). Immunocytochemical staining of keratinocyte specific intermediate filaments using antibodies against cytokeratin 14 (Harnden & Southgate, 1997) identified the isolated cells predominantly as keratinocytes. The fibroblasts present in the culture flasks (about 5-10% per flask) (Fig. 5 A) could be eliminated using mild trypsin treatment as shown by Linge, *et. al.*, in 1989. Earlier findings show that many G-protein coupled receptors (GPCRs) localize at the cell nucleus where they lead to *in situ* gene induction (Chemtob, *et. al.*, 2010). Likewise, immunocytochemical staining of PAR2 was largely detected around nuclei of the cultured primary keratinocytes (Fig. 5). This observation provided an evidence for the presence of PAR2 in reservoirs within successfully isolated primary human keratinocytes. PAR2 were later targeted to study interactions of keratinocytes with P8-modified cyanobacteria, since P8-induced PAR2 activation can result in cellular uptake of the cargo (WO 2019/057988 A1).

The cyanobacteria *Synechococcus elongatus* PCC 7942 used in the experiments described in the Examples were cultivated in 30% osmolarity of ESAW medium with conditions mimicking those found in keratinocyte cytosol including pH, temperature, and salinity. In addition, the survival of *S. elongatus* PCC7942 in cytosol extracts isolated from HaCaT cells was investigates. The obtained results revealed that cyanobacteria PCC 7942 remain viable up to 48 hours (Example 3, Section 4.2) in cytosol extracts isolated from HaCaT cells. Like many other Gram-negative bacteria, the cyanobacteria possess an outer gelatinous slime layer mainly

made up of glycoproteins and glycolipids. It has been demonstrated that the slime layer provides safe environment for cellular growth (Rao, *et. al.*, 2005) which in this case may have protected the cells from cytosolic biomolecules. Decreased fluorescence of the light-harvesting accessory pigments of cyanobacteria was observed at the beginning of incubation which increased over time (Example 3, Section 4.2). Earlier studies have observed fluctuations in the densities of light-harvesting accessory pigments of cyanobacteria in response to stress causing conditions including metal toxicity (Zaccaro, *et. al.*, 2001) or irradiance stress (Macintyre, *et. al.*, 2002). Previously, the composition of phycobiliproteins in cyanobacteria have also been reported to change under stress conditions (Shukla, 2016). Therefore, a reduction observed in PCB fluorescence of cyanobacteria *S. elongatus* PCC 7942 herein may be an indication that applied culture conditions and light intensities have caused stress to the cyanobacteria (Example 3 Section 4.2). It may also be a result of inoculating a new culture from a dense stock culture or altered composition of pigments within the cyanobacteria. A gradual increase in the fluorescence of the phycocyanobilin (Ex638 nm/ Em656 nm) from PCC 7942 revealed the cyanobacteria being in the process of acclimatization to their new environmental conditions as reviewed by Macintyre, *et. al.*, 2002. Therefore, these preliminary findings provide a good evidence that the strain PCC 7942 will remain functional in the cytosol of viable-keratinocytes after their uptake.

In order to achieve conjugation of P8 to surface molecules of cyanobacteria, the coupling reagents EDC and NHS were used to activate carboxyl groups on the surface of cyanobacteria. Resulting NHS-ester intermediates should then react with the amine group of P8 to form the corresponding amide product (Section 3.5). Previous studies have shown that the acidic pH is optimum for efficient EDC coupling (pH 4.5) and the reaction efficiency reduces at neutral pH (7.0 - 7.4), and in phosphate buffers which can be compensated by increasing amount of EDC in the reaction (Mattson, *et. al.*, 1993). Addition of large excess of NHS to the reaction has been studied to convert the unstable o-acylisourea intermediate to a stable amine-reactive NHS-ester intermediate while minimizing undesirable side products (Mattson, *et. al.*, 1993). Earlier, we demonstrated that the use of EDC and NHS at concentrations of 4 µM and 2 µM lead to successfully functionalize silicon dioxide nanoparticles. Our experiments using the same concentration (4 µM) and a five-fold higher concentration (20 µM) of both EDC and NHS revealed no adverse effects on functionality of *S. elongatus* PCC 7942 cells in a detectable way. Hence, the final EDC-NHS reaction protocol to be used on *S. elongatus* PCC 7942 was optimized at pH 7.4 in PBS, using 10 µM EDC and 20 µM NHS, at room temperature. This

can lead to minimised potential threats to cyanobacteria PCC 7942 including acidification of cytoplasm, alteration of proteins or compositions of photosynthetic pigments, while aiming to maintain the crosslinking reaction efficiency.

In the subsequent steps, chemical P8-conjugation to cyanobacteria was attempted using the above-described protocol. Flow cytometry analysis revealed an association between PAR2-agonist P8 and cyanobacteria PCC 7942 by fluorescence detections at Em517 nm and Em656 nm, respectively. (Example 3, Section 4.4.2). Flow cytometry being a technique with higher resolution power for fluorescence detection relative to fluorescence microscopy (Zola, 2004) could identify the fluorophore-attached P8 molecules (Ex488 nm/Em517 nm) to be located with cyanobacteria PCC 7942 (Example 3, Section 4.4.2) whereas P8 couldn't be detected in the same samples under a fluorescence microscope (*i.e.* the less sensitive technique) at given fluorescence range. Further investigations in the direction of eliminating unbound P8 indicated that the chemically-modified cyanobacteria PCC 7942 were associated with P8 as a combination of covalently attached as well as simply adsorbed P8. Hence, this strategy was demonstrated to be efficient for surface modifying the cyanobacteria PCC 7942.

Further studies shed light on the interactions between P8-conjugated cyanobacteria PCC 7942 and HaCaT cells. Interestingly, their interactions were observed to significantly increase in the presence of covalently-conjugated P8 (Fig. 14) which matched with previous studies performed on PAR2 expressing HaCaT cells stimulated with P8-modified iron oxide nanoparticles. Accumulation of nanoparticles was observed even after thorough washing the cells due to their uptake into cultured keratinocytes (WO 2019/057988 A1).

However, the direct comparison between interaction studies of P8-modified nanoparticles and P8-modified cyanobacteria with keratinocytes should consider factors such as size, complexity of surface molecules as potential sites for P8-conjugation or the ability of P8 to simply adsorb on the surface of cyanobacteria. Hence, further detailed endocytosis assays introduced various control conditions to evaluate the interaction pattern with respect to mode of P8-attachment to the surface molecules of cyanobacteria (Example 5, Section 4.5.2). Notable observation was that the interaction efficiency of cyanobacteria PCC 7942 and keratinocytes was unaffected when P8 was added in the absence of coupling reagents. This in turn suggested the importance of chemical conjugation of P8 to cyanobacteria PCC 7942 prior to their addition to the HaCaT cells. Since simple adsorption of P8 couldn't contribute to increased interactions, the successful

covalent conjugation of P8 to cyanobacteria PCC 7942 using the optimised protocol provides an orientation of P8 that allows it to bind to and activate PAR2. It is speculated that the possibility of P8 to orient in a manner specific to interact with PAR2 is less likely in the absence of coupling reagents resulting in gradually dissociating P8 (Example 5, Section 4.5.3) from the surface of cyanobacteria PCC 7942.

Using CLSM, cellular localisation of such P8-conjugated cyanobacteria PCC 7942 interacting with the HaCaT cells was further investigated. Cyanobacteria may be located outside or even inside of the cells as a result of PAR2-mediated uptake by the HaCaT cells. Previously, Agapakis *et. al.* successfully generated viable endosymbiotic chimeric tissues. They genetically engineered *S. elongatus* with invasion protein from *Yersinia pestis* to invade cultured mammalian cells (Agapakis, *et. al.*, 2011). These previous studies show the possibility of cyanobacteria to enter and stay functional within mammalian cells. They in turn support the demonstration herein of P8-conjugated cyanobacteria PCC 7942 beeing localised within the cytosol of keratinocytes as observed from CLSM analysis (Fig. 18). Along this line the inventors observed P8-conjugated NPs accumulated in intracellular vesicles and described it in WO 2019/057988 A1. The inventors concluded that enhanced cellular uptake of P8-modified NPs by keratinocytes and their localization in microvesicles, such as endosomes or lysosomes. Interestingly, in the experiments described herein this peculiar phenomenon could be observed as well in a few cases that showed accumulation of multiple P8-modified cyanobacteria (Fig. 18), which may be present inside a vesicle formed as a result of PAR2 activation by P8. Hence, it can be concluded that the experiments described herein successfully demonstrated the P8-mediated enhanced uptake P8-conjugated cyanobacteria PCC 7942 by HaCaT cells without introducing genetic manipulations. Since fluorescence of P8 on accumulated cyanobacteria could not be observed, it can be predicted that either the association of P8 with cyanobacteria is lower as a result of the higher molecular complexity of the cyanobacteria surface relative to that of the NPs used, or the efficiency of internalization of cyanobacteria PCC 7942 by keratinocytes is lower considering their bigger size than that of the NPs.

In view of the experiments described herein are of preliminary nature, however, imply several potential improvements to overcome potential limiting factors. The covalent conjugation between P8 and cyanobacteria PCC 7942 can be optimised by using sulfo-NHS instead of NHS in order to increase water-solubility of products. In the photometric analysis for P8 measurement biological and technical replicates can be introduced (Section 4.5.3). More

accuracy in the viability measurements of cyanobacteria and keratinocytes can be gained by performing trypan blue dye exclusion test or propidium iodide (PI) staining before flow cytometric analysis. Propidium iodide (PI) is a membrane impermeant fluorescent dye which is excited at 488 nm. It excludes the viable cells and binds to double stranded DNA of dead cells (Crompton, *et. al.*, 1992).

In conclusion, the cyanobacteria *Synechococcus elongatus* PCC 7942 were chemically surface-modified with P8 molecules. The conditions for P8 attachment were established and their association was investigated. Since P8 was found to be present as covalently bound as well as adsorbed, more studies can be conducted if the degree of covalent conjugation of P8 with cyanobacteria PCC 7942 is of particular interest.

Further, an assay for interaction studies between human keratinocytes and chemically P8-conjugated cyanobacteria PCC 7942 has been established. P8 depending interactions were demonstrated and the P8-conjugated cyanobacteria PCC 7942 were identified to internalize by the HaCaT cells. The uptake of cyanobacteria PCC 7942 by keratinocytes can be also investigated by performing scanning electron microscopy to identify localization of cells of cyanobacteria. Live-cell imaging of the cultures can indicate vitality of both cell types. Utilizing cellular markers to specifically detect endosomal proteins in the immunocytochemical staining may locate cyanobacteria potentially present within an endosome. P8-mediated endocytosis of cyanobacteria PCC 7942 into PAR2 expressing cells can be optimized, *e.g.*, by the use of proteases like trypsin or antibiotics such as gentamycin to eliminate non-internalized cyanobacteria within the cultures. In order to quantify oxygen production and reactive oxygen species as a consequence of cyanobacterial photosynthesis activity within the cytosol of living keratinocytes, we will use our microfluidic-based oxygen sensing platform (Bunge *et al.* 2019).

In summary, the experiments provided in the Examples above provide a method for a non-genetic approach of enhanced cellular uptake of micro vesicles but also of complex structures, such as structures with complex and potentially not completely known surface compositions, such as living microorganisms into PAR and in particular PAR2 expressing host cells for the generation of endosymbiotic biological systems respective cells (or altered host cells). The targeted and controlled delivery of drug-carrier vesicles to the affected cells in a wide variety

of chronic and acute illnesses, such as cancer, pulmonary tuberculosis, Parkinson's disease, and Alzheimer's disease can be investigated. The methods described herein can be used for targeted uptake of viruses, virus-like particles or micro-organisms by cells for research or therapeutic purpose. Current strategies of vaccine production may be improved by introducing antigen carrying liposomes into target cells using PAR2-P8 system for enhanced uptake and vaccine production.

Example 6: Wound models

Different wound models for skin wound healing and regeneration exist with different complexities. Skin is a three-dimensional, multicellular organ with many functions - first of all protection of the body.

Hence, various types of skin cells respond in an orchestrated way to multiple physiological and pathological stimuli, *e.g.* wounding, by secreting pro-inflammatory factors which in turn can induce activation of cells localised in the surrounding tissue. Quiescent cells adapt an activated status that ensures cellular migration and proliferation which is important for wound closure. To study these processes, well-established *in vitro* cell culture, *ex vivo* organ and *in vivo* skin wound models are available but varying in complexity.

Different experimental models for skin wound repair are used to show the treatment capability of the cells provided by the methods described herein, to improve wound healing of chronic, non-healing skin wounds. In particular, skin cells (or pharmaceutic compositions comprising these) which have been altered by the methods described herein by the introduction of cyanobacteria and are capable to produce oxygen under light irrigation will be engrafted to wounds and improve the healing of the wounds while circumventing the local hypoxia conditions associated with chronic wounds by production of oxygen, as summarized below.

Application of keratinocytes to wounds has been demonstrated to improve wound healing[2]. To determine the migration potential of neo-endosymbiotic keratinocytes generated by the methods of the present invention as well as the secretion of proinflammatory cytokines and growth factors the inventors have established *in vitro* and *ex vivo* human skin organ culture models[6] which are used in these experiments. In particular, these methods are used to show the capability of the (human) keratinocytes, which by the method of the present invention took up

one or more cyanobacteria, which, upon exposure to light, produce oxygen and thereby lower or remove completely the requirement of these keratinocytes for oxygen supply from the outside. When engrafted (e.g., sprayed, seeded or placed by other means) onto acute or chronic wounds, these modified skin cells are able to circumvent associated local hypoxia conditions and subsequently enhance wound closure processes also under such adverse conditions.

6.1 Microfluidic-based oxygen sensing platform to monitor oxygen production and consumption by cells in-vitro

A microfluidic-based oxygen sensing platform was developed by the inventors that can be used to monitor changes in the oxygen concentration, caused by the consumption or production of oxygen in the media of cultured cells as a direct indicator for cell metabolism (Bunge et al. 2019). This platform consists of a microfluidic chip with an integrated oxygen-sensitive phosphorescent film, heater and temperature sensor, external optical read-out and 3D-printed holders and housing. The chip with the closed microfluidic chamber is made by clean-room technologies and consists of silicon and glass. An excitation LED and a small and cost-effective Raspberry Pi camera are used to measure the phosphorescent signal. With this system, the concentration of dissolved oxygen can be determined with an accuracy of ± 0.8% (air) for oxygen concentrations between 0 and 26% (air) at any temperature between 23 and 41°C. This microfluidic oxygen platform is suitable to monitor oxygen production/consumption of neo-endosymbiotic keratinocytes under controlled light and culturing conditions.

The oxygen sensor is further refined to readout parameters specifically tailored to particular requirements being obligatory for further characterization of the neo-endosymbiotic skin cells. Such an improved device will allow for systematic validation of the methods described herein. A refined prototype of the microfluidic-based oxygen sensing platform has already been developed and is tested with different eukaryotic cell types and cyanobacteria. Preliminary data on light induced oxygen production of cyanobacteria as well as oxygen consumption by keratinocytes successfully demonstrated the general functionality of the micro-sensor. The cells produced by the methods of the present invention are tested according to the methods described in said article by Bunge et. al. (2019).

6.2 Cell scratch in vitro wound model in monocultures to study 2D cellular performance with migration, proliferation and secretion of pro-inflammatory mediators

Scratch wounding of cell cultures are performed as described previously [7] to mimic a simple mechanical wound. For the whole experimental description, see, in particular sections "Keratinocyte and fibroblast isolation" and "Scratch tests of keratinocyte or fibroblast monocultures." in the Methods part of this publication[7]. In brief, a scratch through a monolayer of cultured keratinocytes is made mechanically with a micropipette tip, to resembles an *in vivo* wound. Time-resolved cell migration of peripheral cells into the cell free area is captured, digitised and the area *A* of the remaining gap is determined [8]. The ratio *A/A0* is plotted against time, where *A0* is the area at time point 0 post-scratch and *A* is the area at different time points. The smaller the quotient *A/A0* the faster the "wound" is closing. This simple wound closure assay is used to show the capability of neo-endosymbiotic keratinocytes obtained by the methods described herein and applied to the scratch regions in comparison to not treated scratches to enhance cell migration and proliferation of cultured keratinocytes under selected cell culture conditions *in vitro*.

6.3 Ex vivo skin organ culture wound model to study 3D cellular resurfacing

To address a higher experimental complexity, the inventors have established a three-dimensional skin organ culture model [6]. Dermal substrates derived from donated human skin after plastic surgery are used. After defatting, 8-mm punch biopsies are cut out of human skin, the epidermis is stripped off and dermal cells are destroyed by repeated freezing and thawing cycles, which yields the so called de-epidermised dermis (DED). This assay allows for studying cell-cell and cell-matrix interactions in a system with the original morphological and biochemical structure of human skin. For example, the effect of neo-endosymbiotic keratinocytes on migration, secretion of cytokines and growth factors within wound tissue is investigated with this *ex vivo* human skin organ culture model. Normal and endosymbiotic keratinocytes are transferred onto dermal substrates and cultured in complete medium [9] on human DED at the air-liquid-interface.

The exact experiments as performed in this context are described in Mirastschijski et al., Br J Dermatol 154, 42–49 (2006), page 43, section "Tissue culture".

6.4 Excisional in vivo wound healing model in pigs and mice to study wound closure and immunological responses

Complex systemic effects such as systemic immune responses or dermal tissue remodelling require *in vivo* experimental models. Porcine skin resembles human skin with regard to anatomical architecture and physiological immune responses. Previous studies that used this *in vivo* wound model showed that the partial-thickness excisional skin wound on the back of pigs is most suitable for addressing questions such as skin resurfacing and wound contraction. The effect of the neo-endosymbiotic cells on skin wound healing is assessed *in vivo* with a standard experimental model using partial-thickness excisional wounds on the back of mini pigs. In particular, standardized wounds with a depth of 0.3 mm are made with a dermatome on the back of anesthetized animals after disinfection and shaving. Previously harvested and cultured keratinocyte sheets with and without neo-endosymbiotic keratinocytes as generated by the methods of the present invention are transferred onto wounds after hemostasis and periodically illuminated with light prior to application of wound dressings. Wounds are covered with several sheets of fatty gauze and fixed with tape. Time dependent wound closure and wound contraction is analyzed at various time points post-operatively by digital photo-imaging and wound margin tracings. At the end of the experiment, animals are sacrificed, wounds excised and subjected to further analyses including immunohistology and MALDI-IMS. Alternatively the experiments are performed in mice, as described in section "Excisional full-thickness skin wound model in mice." of Mirastschijski et al Scientific Reports 2020.

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Abbreviations

°C	degree Celsius
%	percent
5-FAM	5-carboxyfluorescein
APC	allophycocyanin
Arg; R	arginine
ca.	circa
CK-14	cytokeratin-14
Cy3	cyanine 3
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethylsulfoxide
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
ESAW	enriched artificial sea water medium
et. al.	and others (lat.: *et alii*)

FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
Fmoc	fluorenylmethyloxycarbonyl
FSC	forward scatter
g	gram
Gly; G	glycine
GPCR	G-protein coupled receptor
h	hour
HaCaT	the human adult low calcium high temperature keratinocytes
ICC	immunocytochemistry
Ile; I	isoleucine
KGM2	keratinocyte growth medium 2
L	liter
Leu; L	leucine
Lys; K	lysine
µ	micro
M	molar (mol/L)
mg	milligram
min	minutes
mM	millimolar
NaH_2PO_4	sodium dihydrogen phosphate
$NaNO_3$	sodium nitrate
Na_2SiO_3	sodium silicate
NHS	N-hydroxysuccinimide
NPs	nanoparticles
PBS	phosphate buffered saline
PC	phycocyanin
PCB	phycocyanobilin
PE	phycoerythrin
PEB	phycoerythrobilin
PI	propidium iodide

PSI	photosystem I
PSII	photosystem II
RCF/*g*	relative centrifugal force in g
rpm	revolutions per minute
sec	seconds
Ser; S	serine
SSC	side scatter
UV	ultra violet

CLAIMS

1. A method for providing a cell with a desired phenotypic characteristic, comprising:
   a) incubating a structure provided with a biological or equivalent membrane or envelope and conjugated with a PAR binding ligand with an host cell expressing a protease activated receptor (PAR) on its surface under conditions allowing uptake of the structure into the host cell, wherein the structure or its content is capable of conferring the desired phenotypic characteristic to the host cell; and
   b) detecting the cell with said phenotypic characteristic, and optionally isolating the cell.

2. The method of claim 1, wherein the phenotypic characteristic is other than the presence or level of said structure or its content per se.

3. The method of claim 1 or 2, wherein the PAR binding ligand is conjugated to the structure by a linker molecule, wherein the linker molecule comprises an aromatic moiety.

4. The method of claim 3, wherein the aromatic moiety is a fluorophore such as 6-carboxyfluorescein (6-FAM) und 5-carboxyfluorescein (5-FAM) or a mixture thereof, or eosin, rhodamine or corresponding derivatives.

5. The method of any one of claims 1 to 4, wherein the structure provided with a biological or equivalent membrane or envelope is a cell.

6. The method of claim 4, wherein the cell as structure is a prokaryotic cell.

7. The method of any one of claims 1 to 6, wherein the desired phenotypic characteristic is an effect on production or consumption of a molecule, change of a response of the host cell to an added substance, effect on the ability of the host cell to grow or die in an environment or an observable alteration of a cultural, metabolic, behavioral or morphological characteristic of the host cell or induction of the death of a cell.

8. The method of any one of claims 1 to 7, wherein the host cell is a eukaryotic cell, preferably a mammalian cell.

9. The method of claim 8, wherein the host cell is a keratinocyte, preferably a human keratinocyte.

10. The method of any one of claims 6 to 9, wherein the structure is a cyanobacterium.

11. The method of any one of claims 1 to 10, wherein compared to the initial host cell the phenotypic characteristic is production of oxygen or reduced oxygen consumption by the host cell.

12. Cell obtainable by the method of any one of claims 1 to 11.

13. A composition or kit comprising the cell of claim 12, wherein the composition is a pharmaceutic composition comprising a pharmaceutically acceptable carrier.

14. Cell of claim 12 or composition of claim 13 for use in the treatment of wounds.

15. Use of a PAR binding ligand for the generation of an endosymbiotic cell.

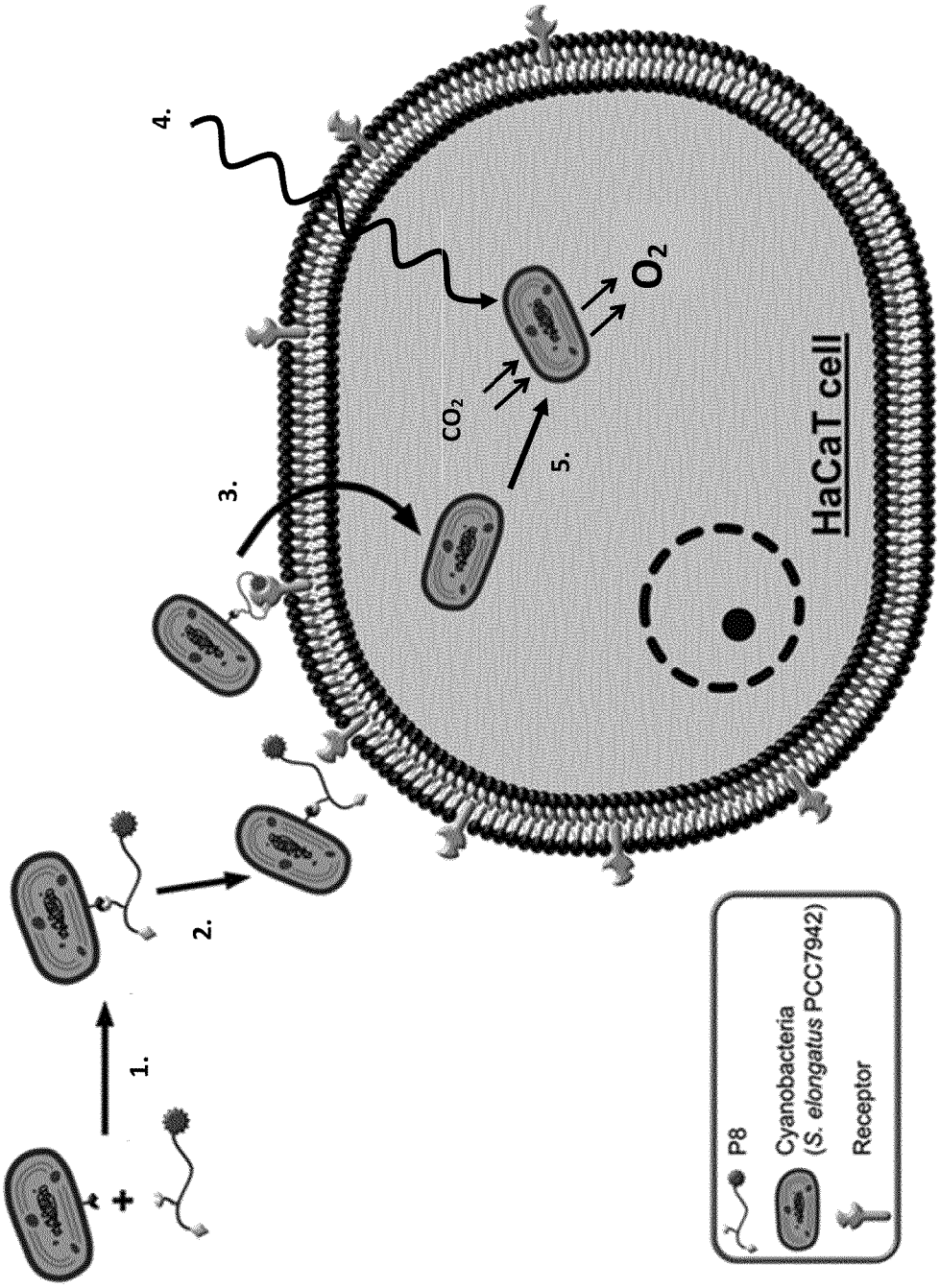

1.
2.
3.
4.
5.
CO_2
O_2
HaCaT cell
P8
Cyanobacteria
(*S. elongatus* PCC7942)
Receptor

Fig. 1

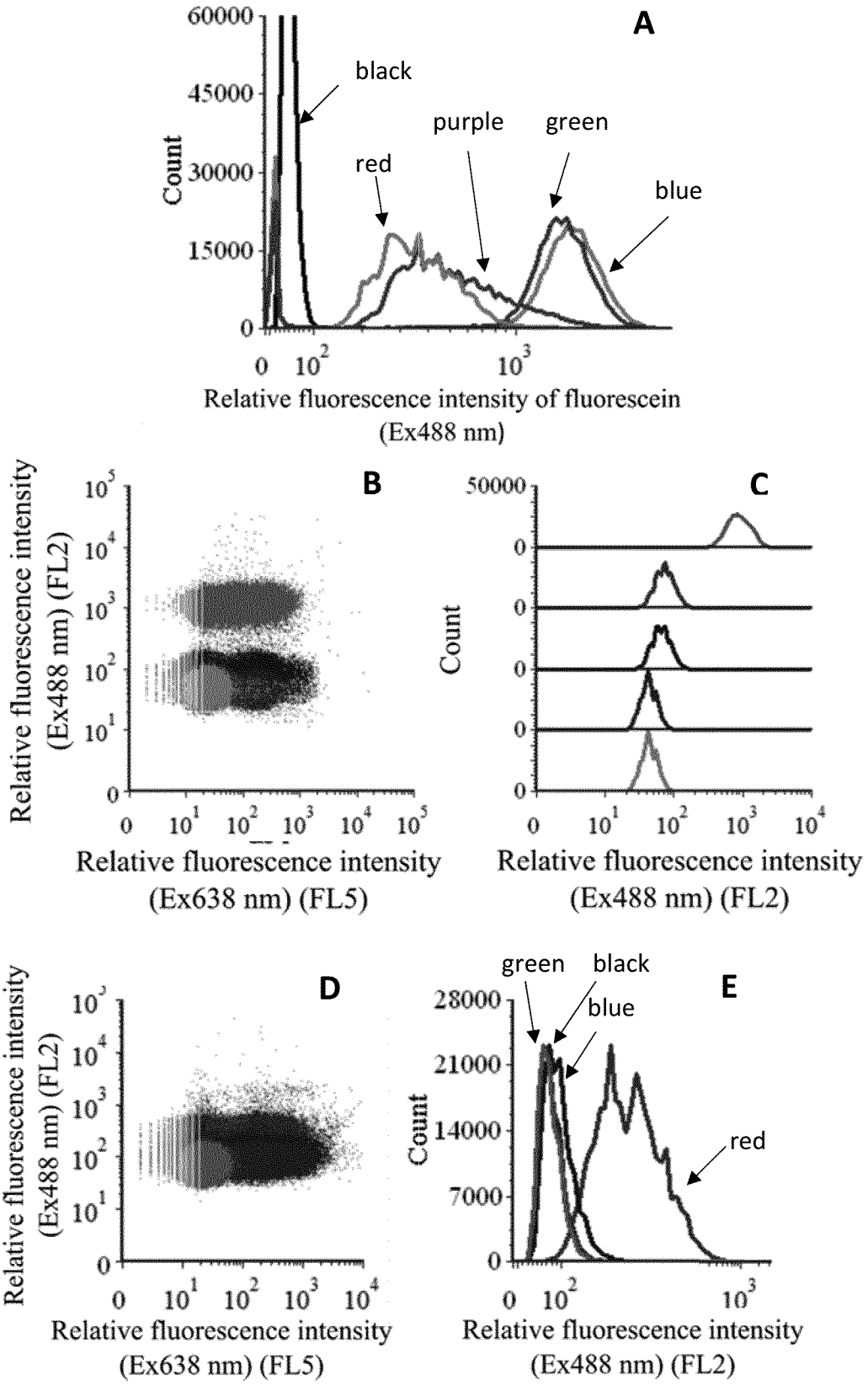

A
black
red
purple
green
blue
Count
60000
45000
30000
15000
0
Relative fluorescence intensity of fluorescein
(Ex488 nm)
B
Relative fluorescence intensity
(Ex488 nm) (FL2)
Relative fluorescence intensity
(Ex638 nm) (FL5)
C
Count
Relative fluorescence intensity
(Ex488 nm) (FL2)
D
Relative fluorescence intensity
(Ex488 nm) (FL2)
Relative fluorescence intensity
(Ex638 nm) (FL5)
E
green
black
blue
red
Count
28000
21000
14000
7000
Relative fluorescence intensity
(Ex488 nm) (FL2)

Fig. 2

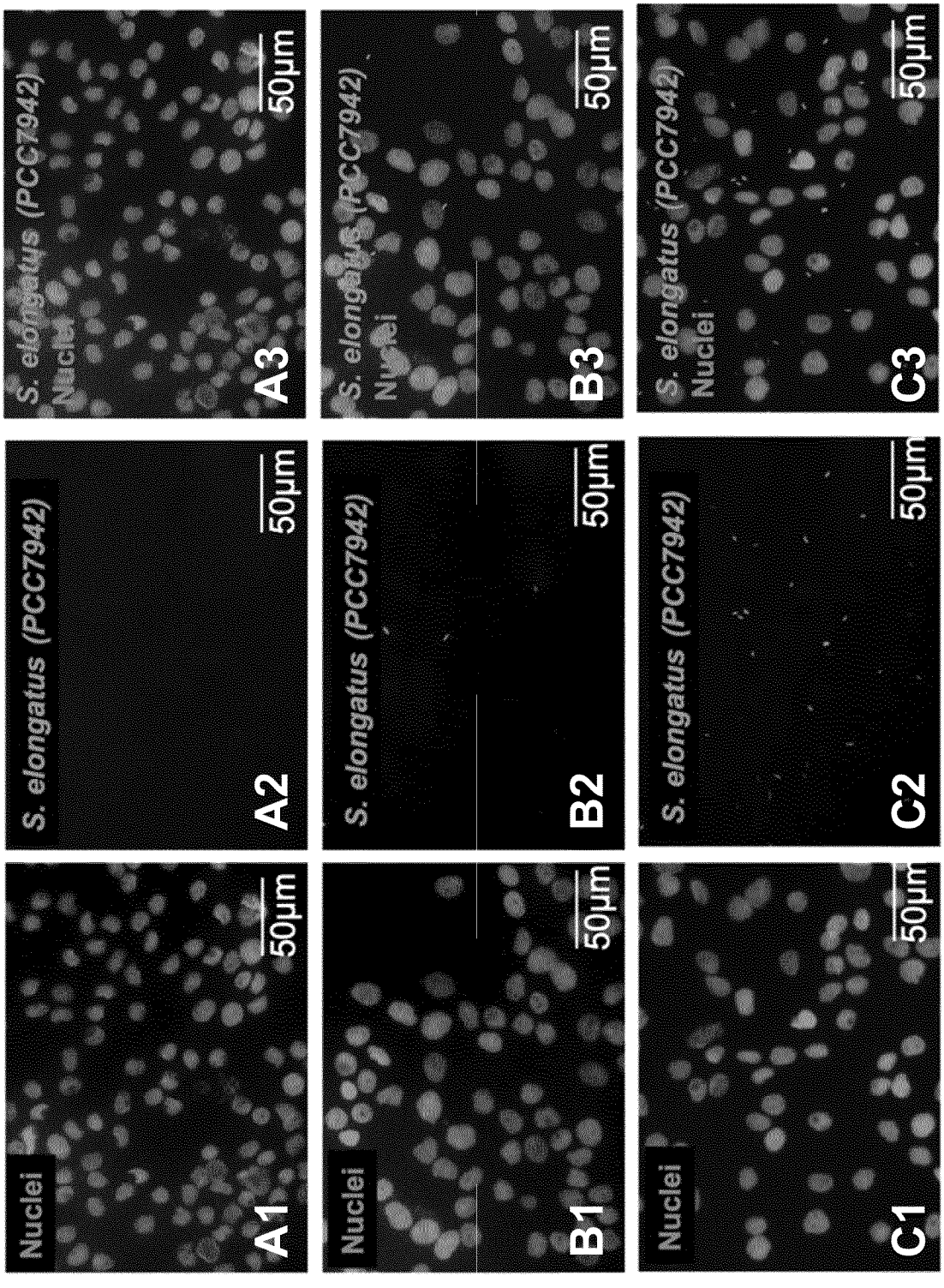

Nuclei
A1
50µm
S. elongatus (PCC7942)
A2
50µm
S. elongatus (PCC7942)
Nuclei
A3
50µm
Nuclei
B1
50µm
S. elongatus (PCC7942)
B2
50µm
S. elongatus (PCC7942)
Nuclei
B3
50µm
Nuclei
C1
50µm
S. elongatus (PCC7942)
C2
50µm
S. elongatus (PCC7942)
Nuclei
C3
50µm
HaCaT + PCC7942
HaCaT + PCC7942 + P8
HaCaT + PCC7942(P8)

Fig. 3

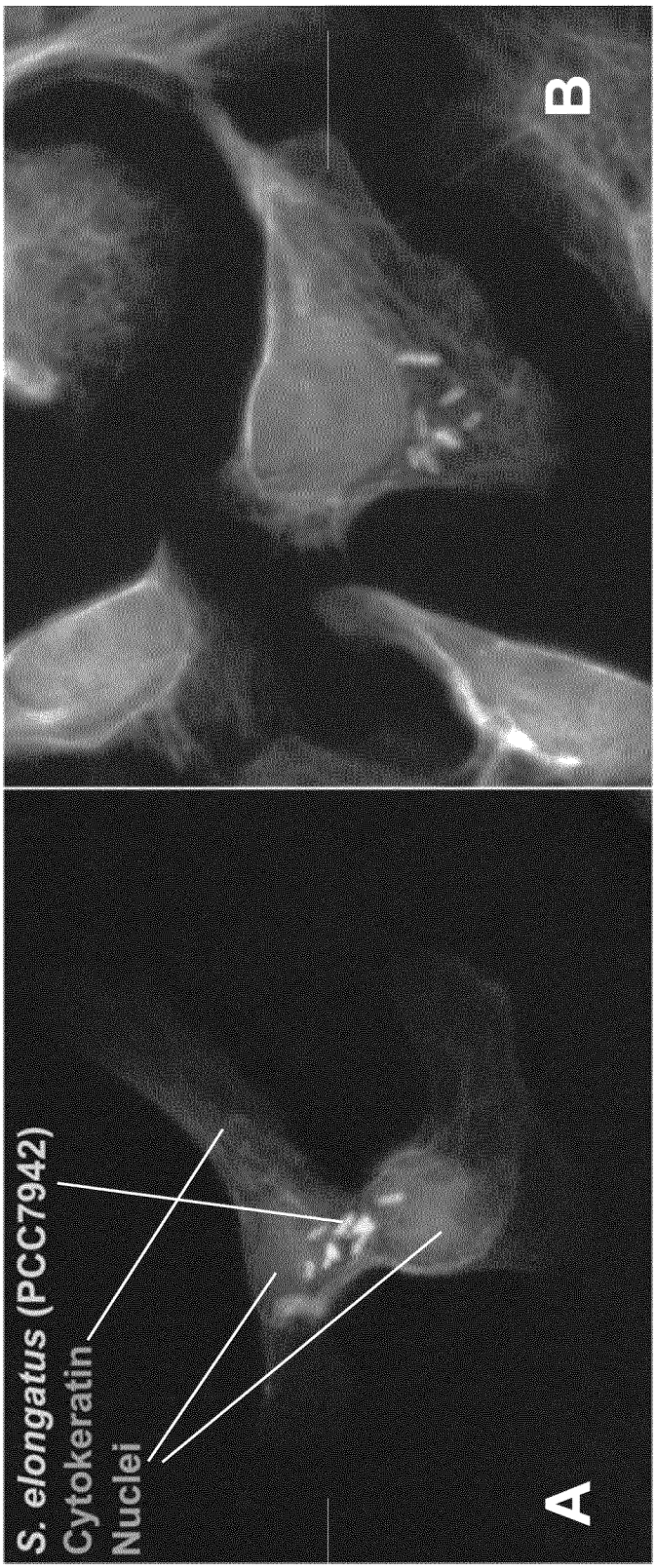


Fig. 4

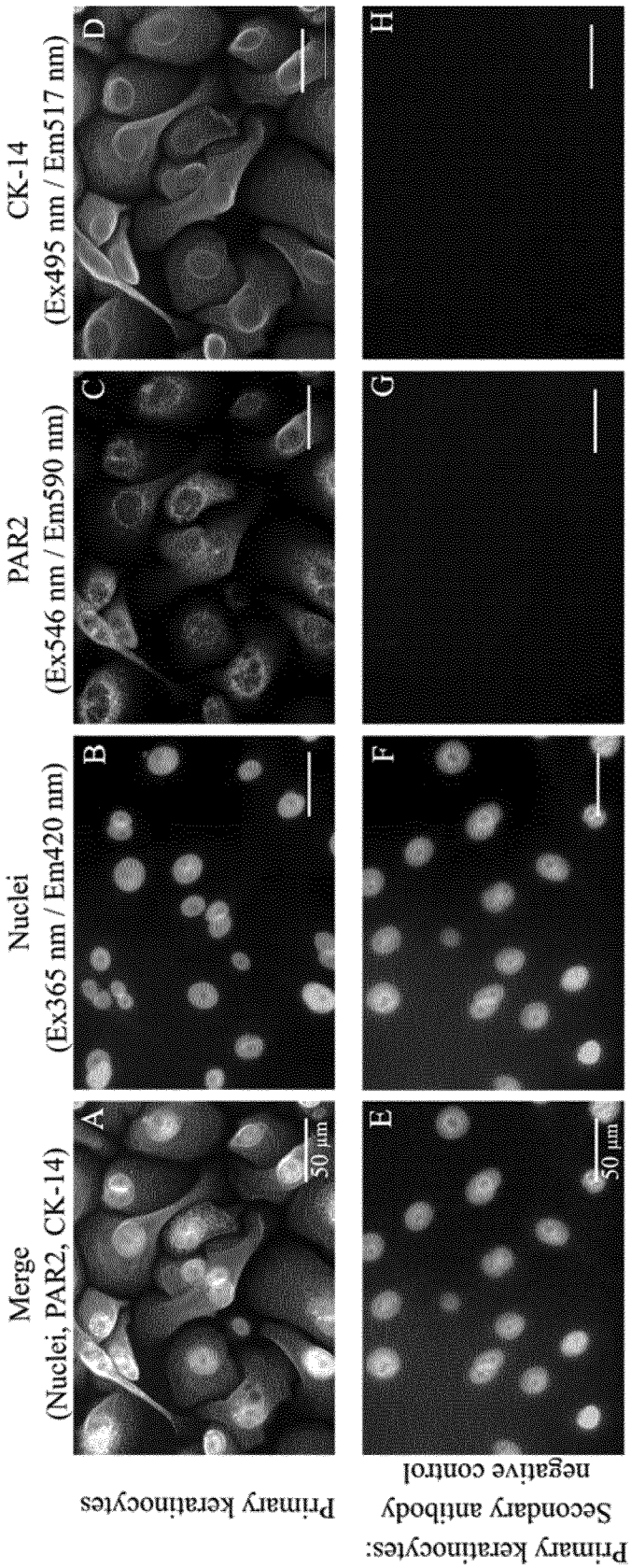
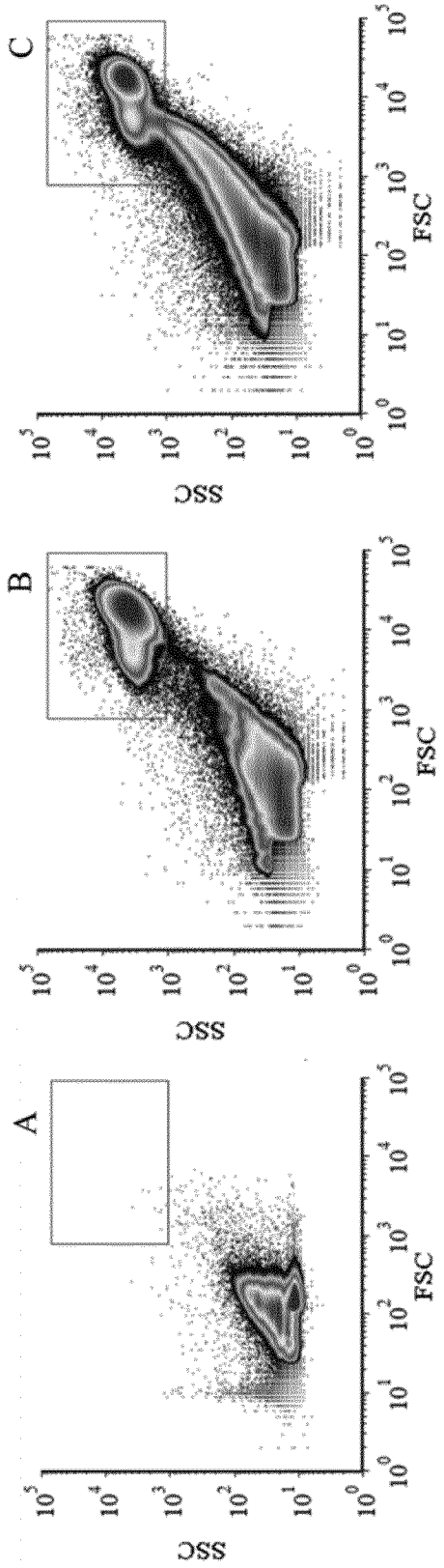

Merge
(Nuclei, PAR2, CK-14)
Nuclei
(Ex365 nm / Em420 nm)
PAR2
(Ex546 nm / Em590 nm)
CK-14
(Ex495 nm / Em517 nm)
A
B
C
D
E
F
G
H
50 µm
50 µm
Primary keratinocytes
Primary keratinocytes:
Secondary antibody
negative control

Fig. 5



Fig, 6

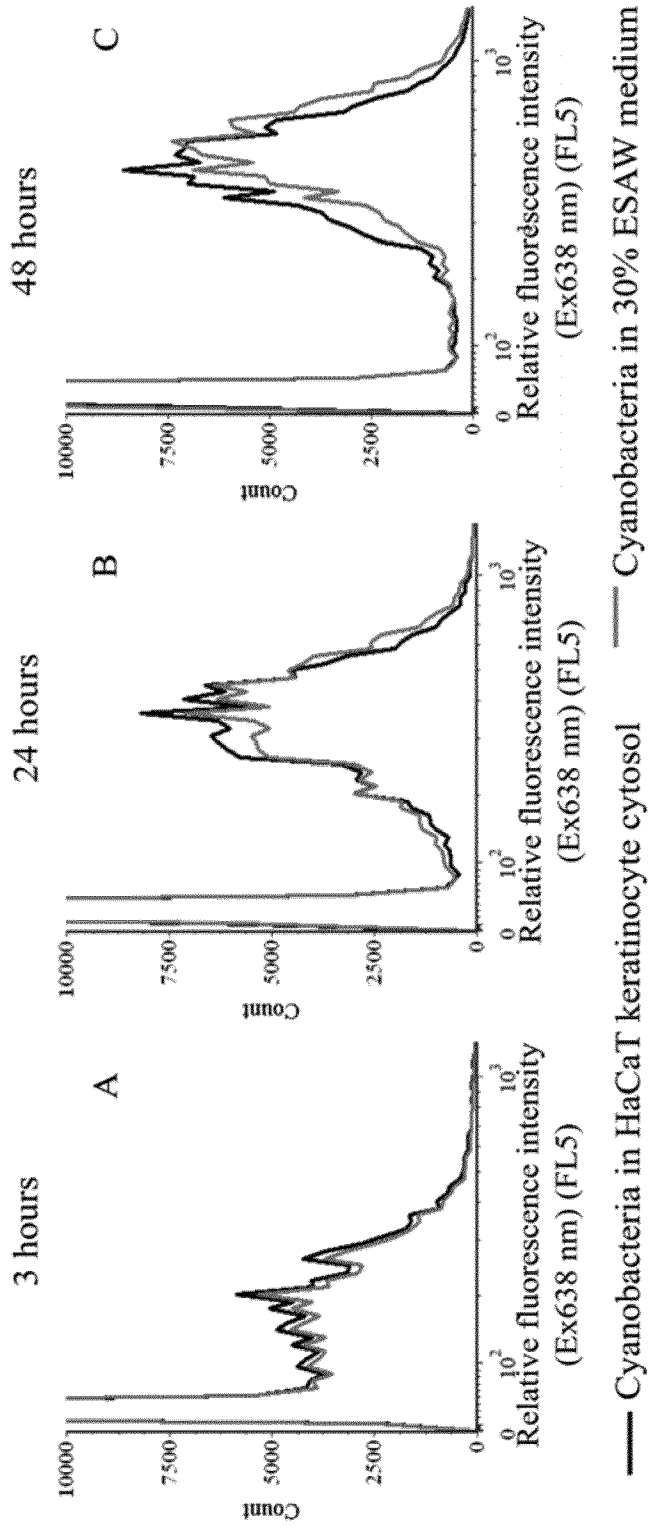

3 hours
A
24 hours
B
48 hours
C
Count
Relative fluorescence intensity
(Ex638 nm) (FL5)
Cyanobacteria in HaCaT keratinocyte cytosol
Cyanobacteria in 30% ESAW medium

Fig. 7

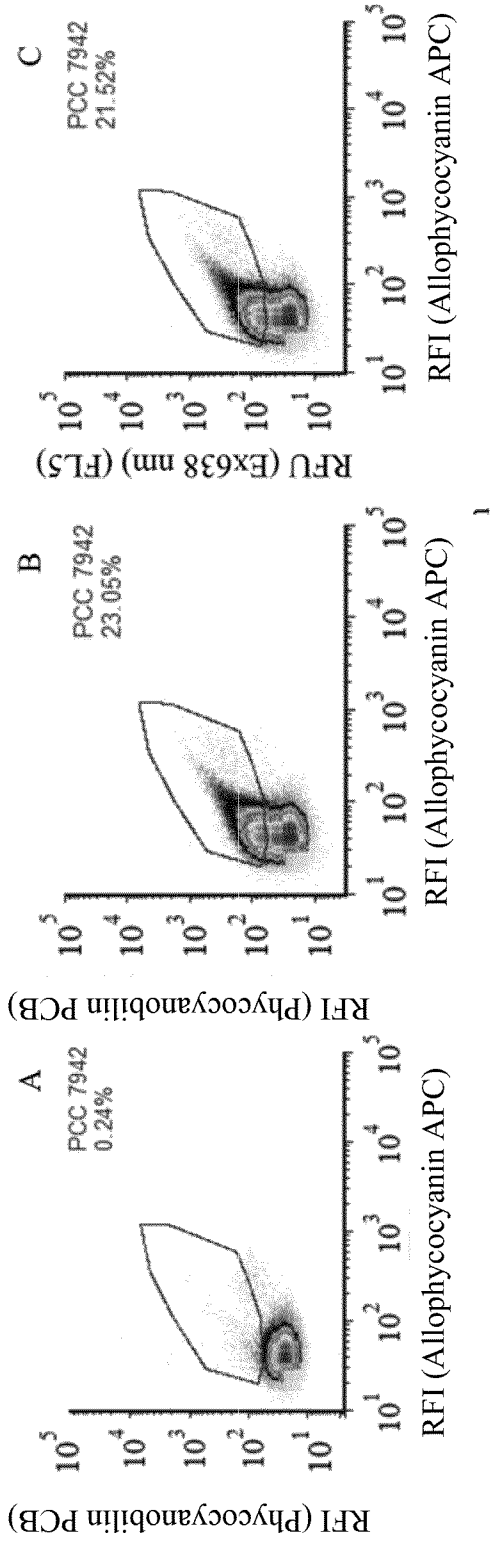
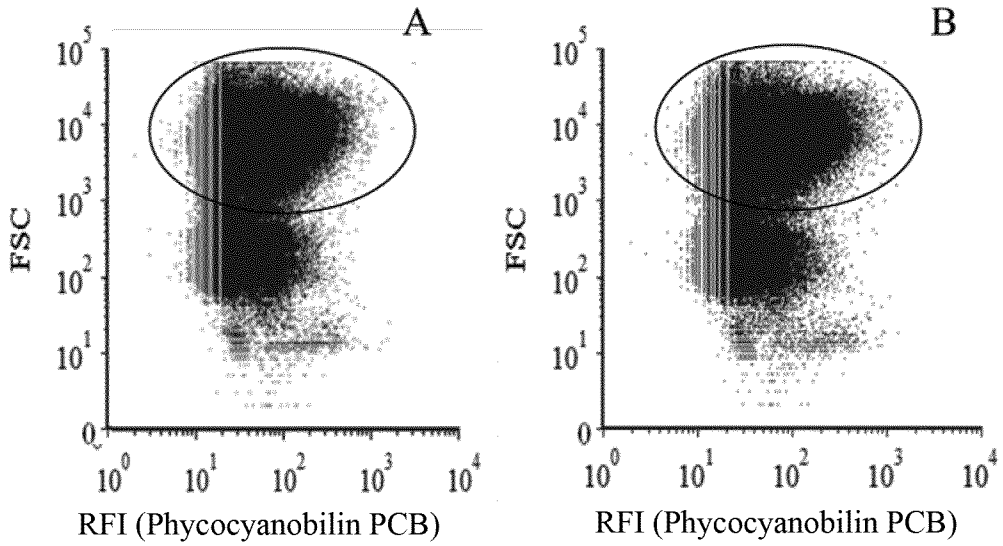

A
PCC 7942
0.24%
B
PCC 7942
23.05%
C
PCC 7942
21.52%
RFI (Phycocyanobilin PCB)
RFI (Allophycocyanin APC)
RFU (Ex638 nm) (FL5)

Fig. 8


A
B
FSC
RFI (Phycocyanobilin PCB)
RFI (Phycocyanobilin PCB)

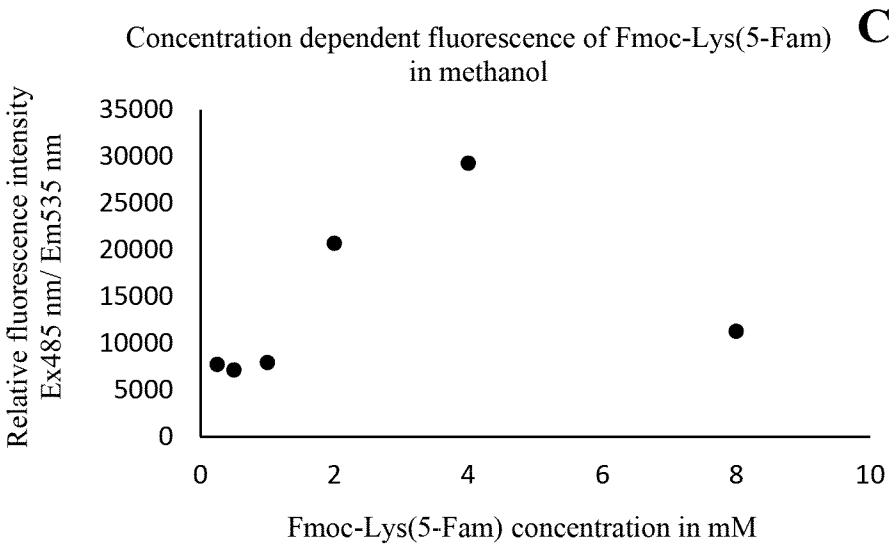

C
Concentration dependent fluorescence of Fmoc-Lys(5-Fam) in methanol
Relative fluorescence intensity Ex485 nm/ Em535 nm
Fmoc-Lys(5-Fam) concentration in mM

Fig. 9

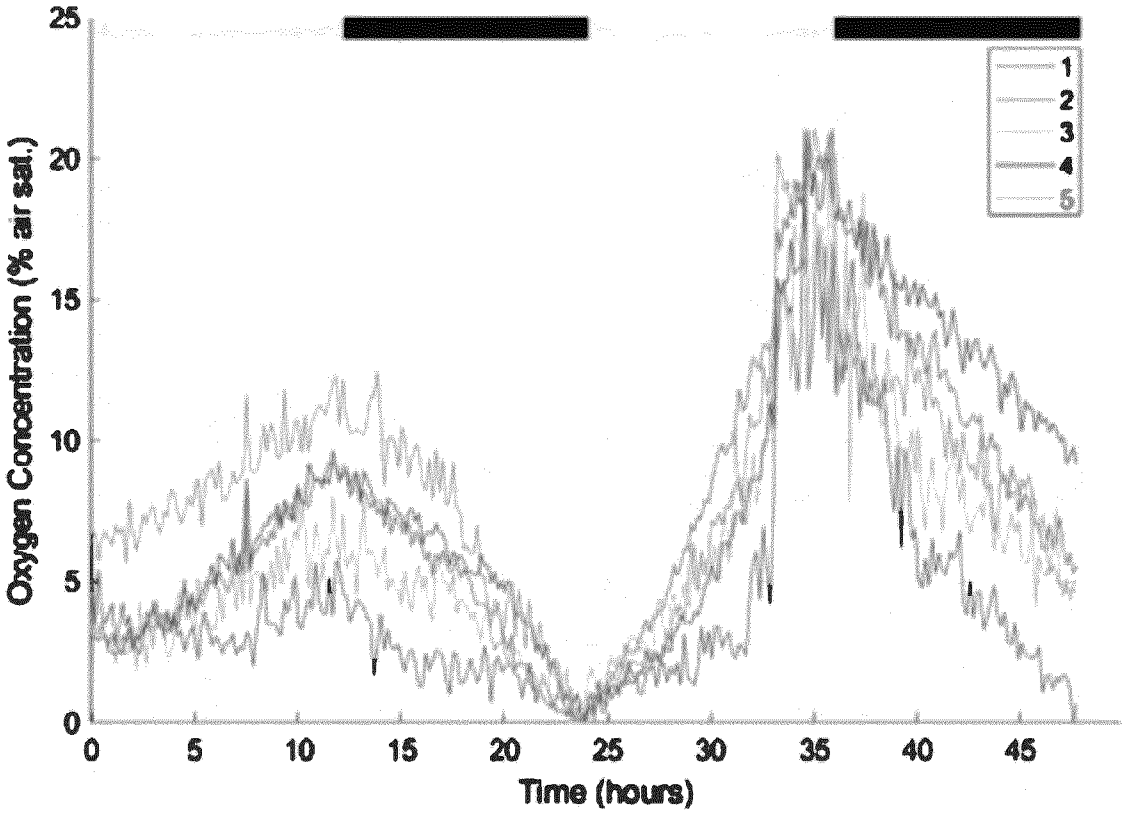

Oxygen Concentration (% air sat.)
Time (hours)
1
2
3
4
5
0
5
10
15
20
25
30
35
40
45

Fig. 10

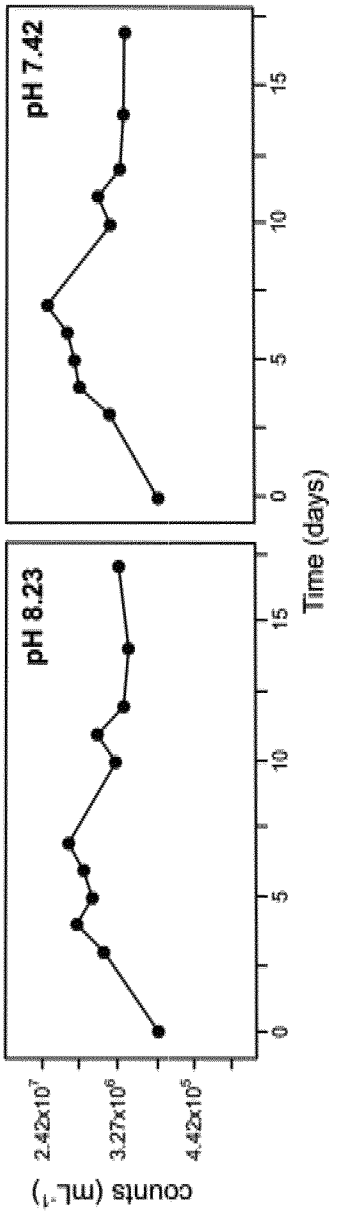

pH 7.42
pH 8.23
0
5
10
15
Time (days)
2.42x10^7
3.27x10^6
4.42x10^5
counts (mL^-1)

Fig. 11

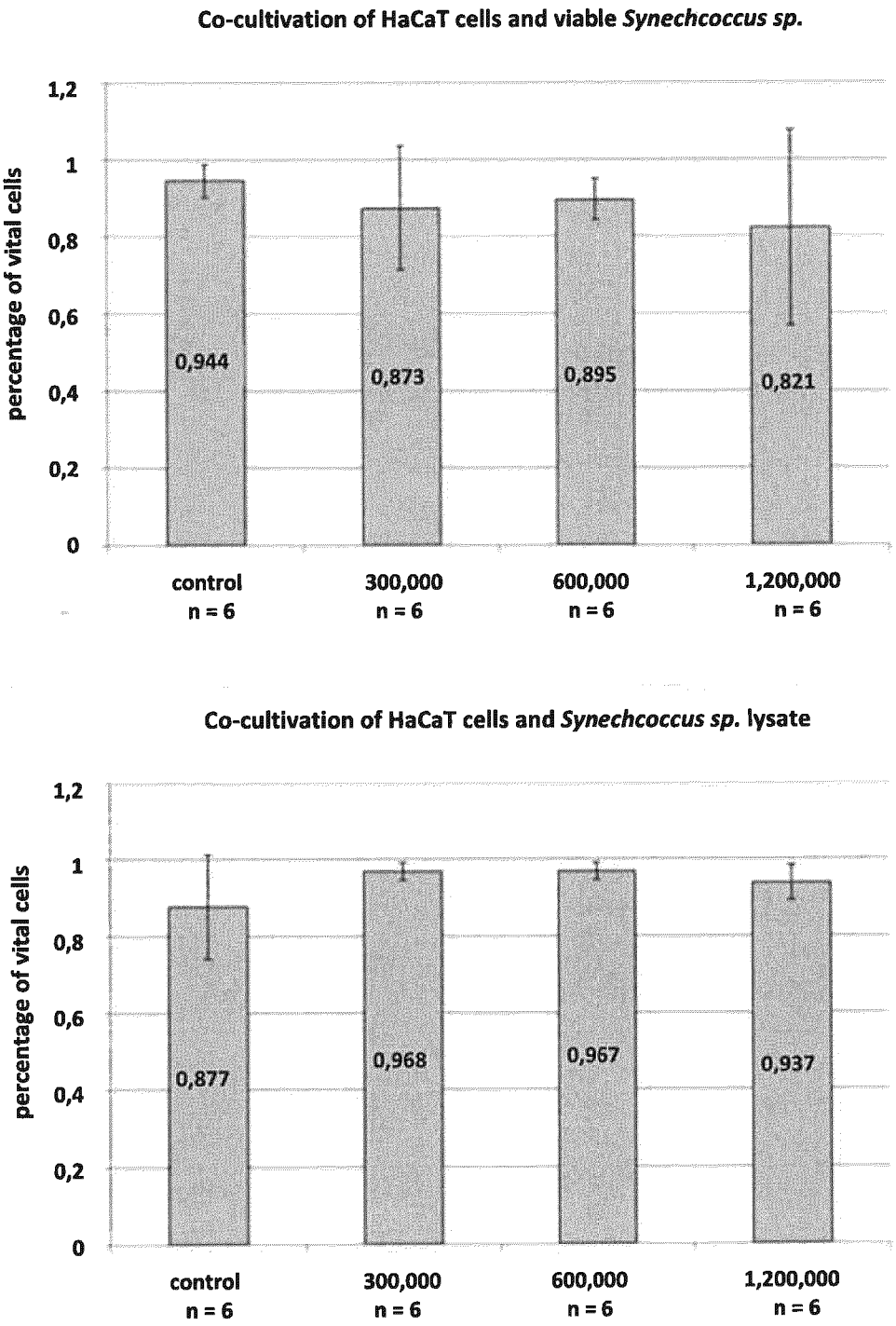

Co-cultivation of HaCaT cells and viable *Synechcoccus sp.*
percentage of vital cells
1,2
1
0,8
0,6
0,4
0,2
0
0,944
0,873
0,895
0,821
control n = 6
300,000 n = 6
600,000 n = 6
1,200,000 n = 6
Co-cultivation of HaCaT cells and *Synechcoccus sp.* lysate
percentage of vital cells
1,2
1
0,8
0,6
0,4
0,2
0
0,877
0,968
0,967
0,937
control n = 6
300,000 n = 6
600,000 n = 6
1,200,000 n = 6

Fig. 12

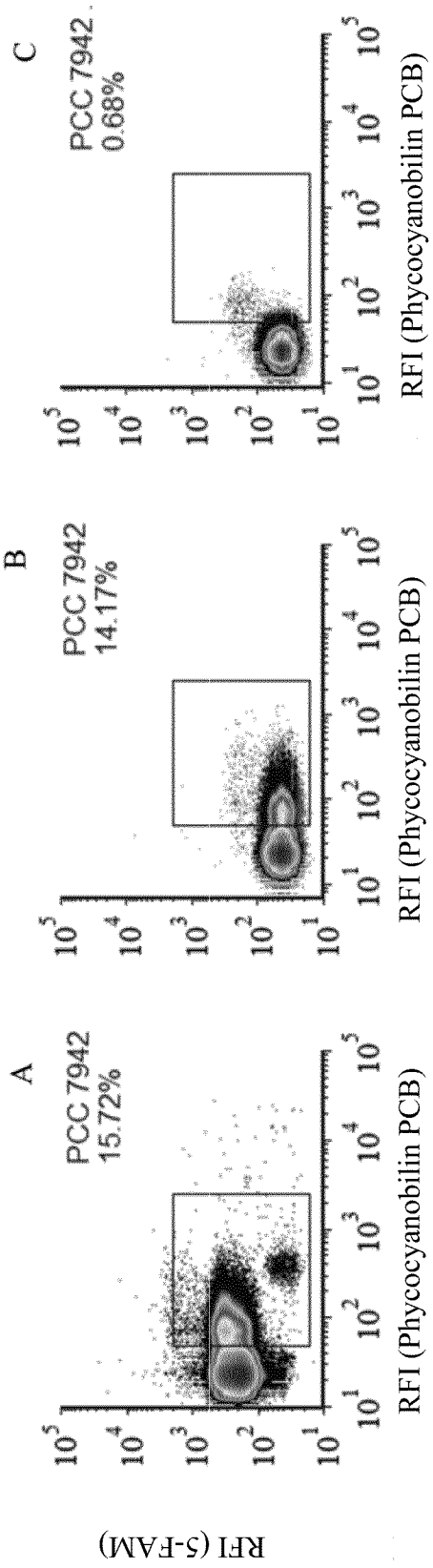

A
PCC 7942
15.72%
B
PCC 7942
14.17%
C
PCC 7942
0.68%
RFI (Phycocyanobilin PCB)
RFI (5-FAM)

Fig. 13

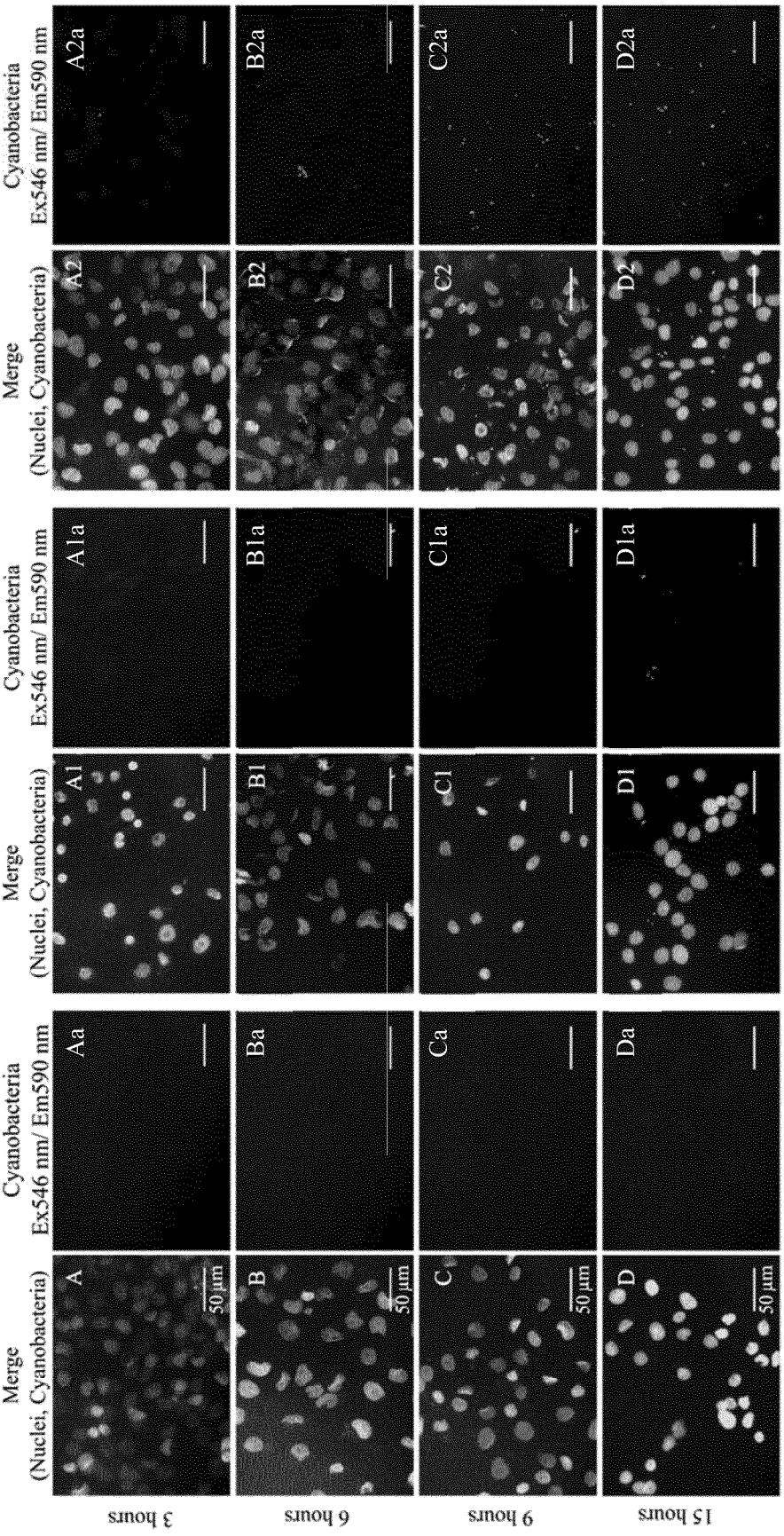

Merge
(Nuclei, Cyanobacteria)
Cyanobacteria
Ex546 nm/ Em590 nm
Merge
(Nuclei, Cyanobacteria)
Cyanobacteria
Ex546 nm/ Em590 nm
3 hours
6 hours
9 hours
15 hours
A
Aa
A1
A1a
A2
A2a
B
Ba
B1
B1a
B2
B2a
C
Ca
C1
C1a
C2
C2a
D
Da
D1
D1a
D2
D2a
50 µm

Fig. 14

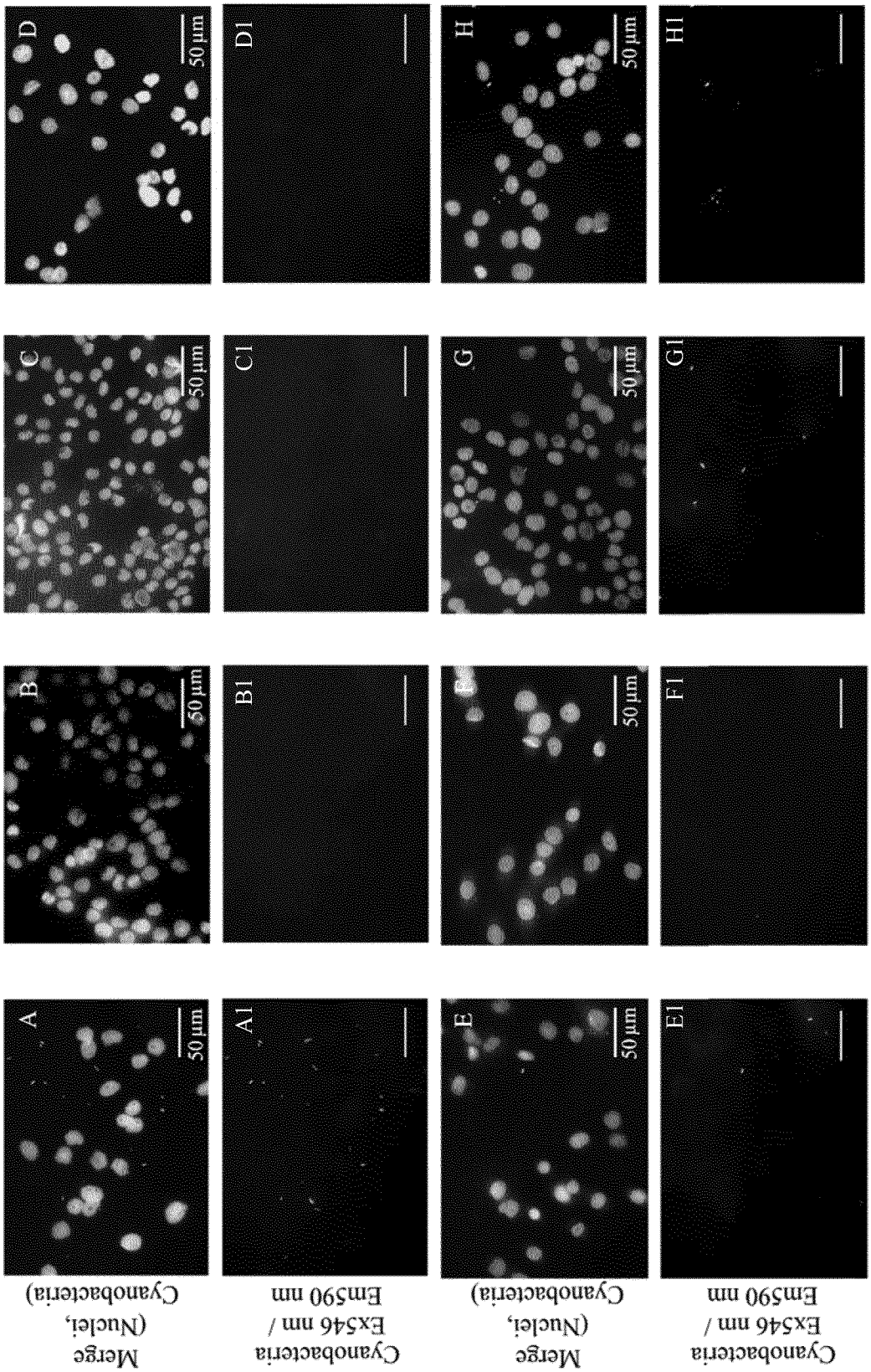

A
50 µm
A1
E
50 µm
E1
B
50 µm
B1
F
50 µm
F1
C
50 µm
C1
G
50 µm
G1
D
50 µm
D1
H
50 µm
H1
Merge
(Nuclei,
Cyanobacteria)
Cyanobacteria
Ex546 nm /
Em590 nm
Merge
(Nuclei,
Cyanobacteria)
Cyanobacteria
Ex546 nm /
Em590 nm

Fig. 15

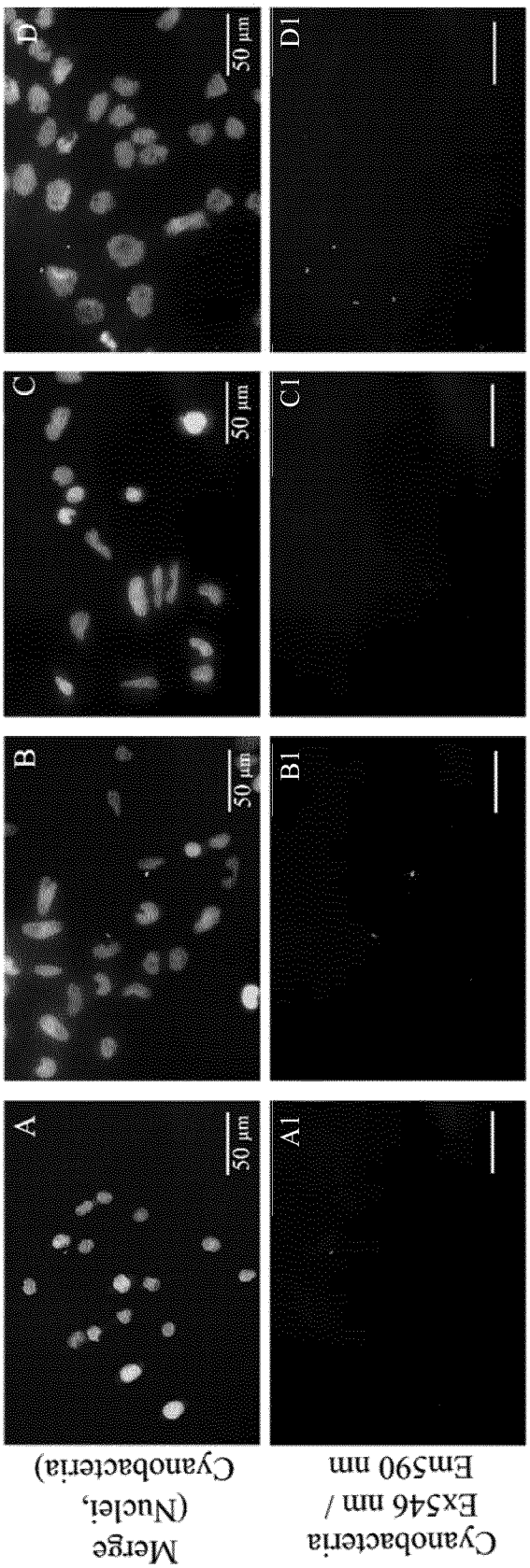

A
B
C
D
A1
B1
C1
D1
50 µm
Merge
(Nuclei,
Cyanobacteria)
Cyanobacteria
Ex546 nm /
Em590 nm

Fig. 16

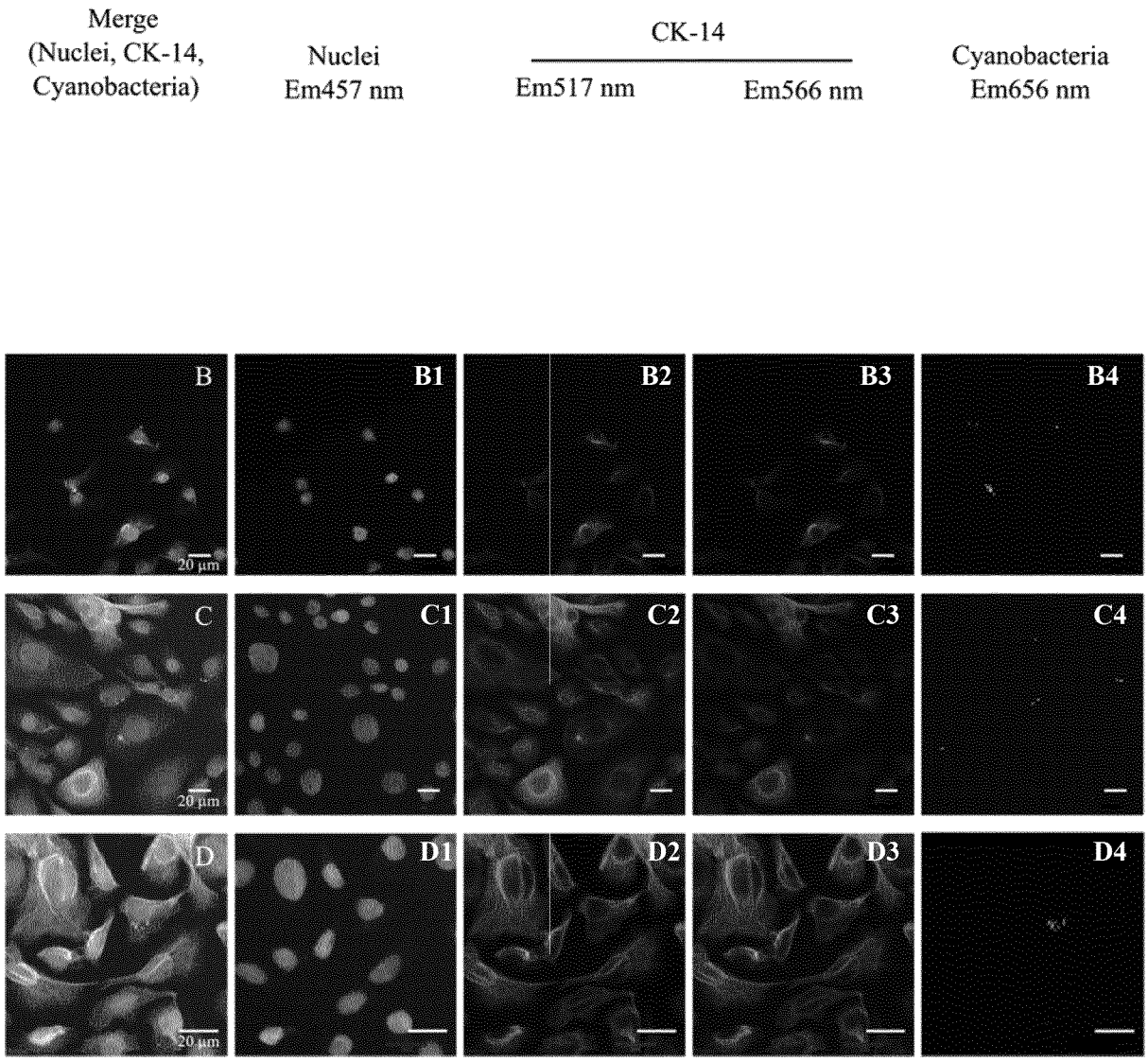

Merge
(Nuclei, CK-14,
Cyanobacteria)
Nuclei
Em457 nm
CK-14
Em517 nm
Em566 nm
Cyanobacteria
Em656 nm
B
B1
B2
B3
B4
C
C1
C2
C3
C4
D
D1
D2
D3
D4
20 µm

Fig. 17

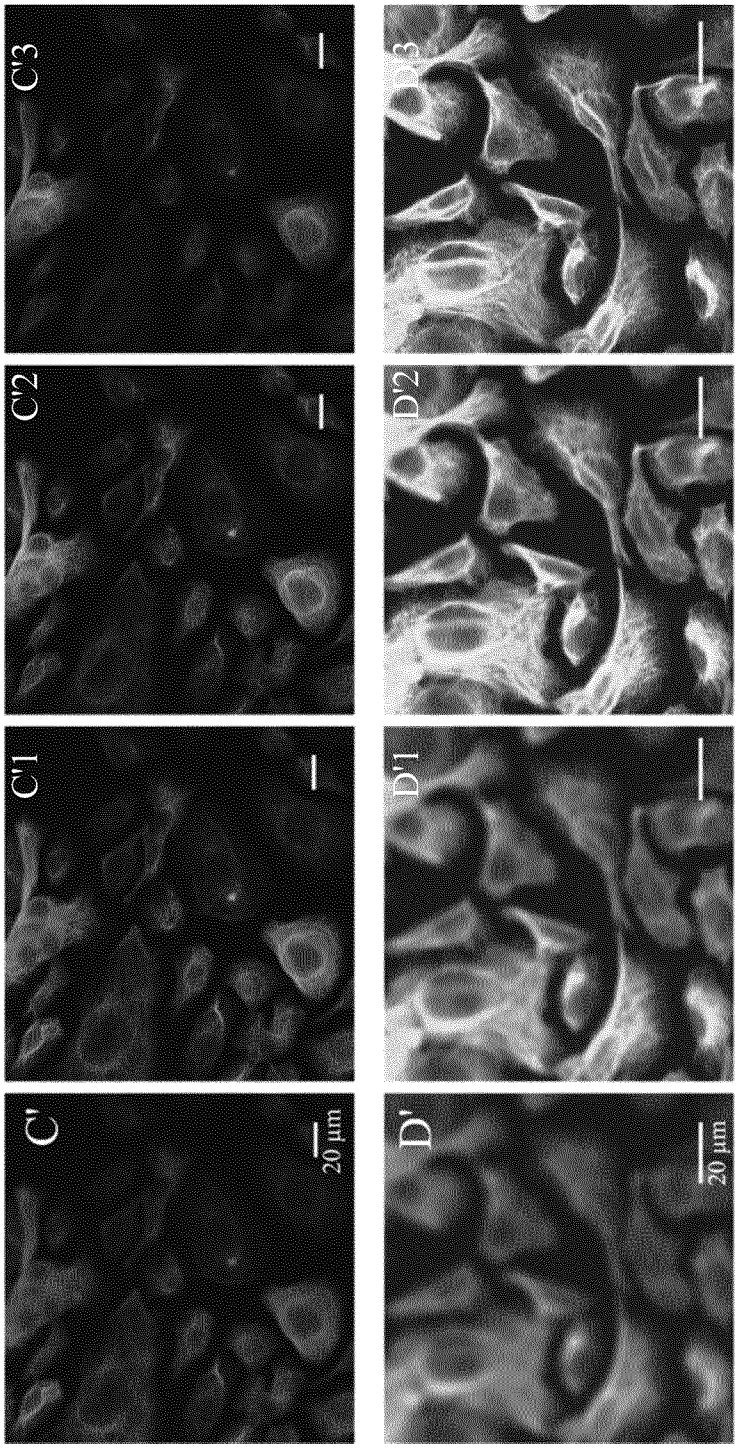


Fig. 18

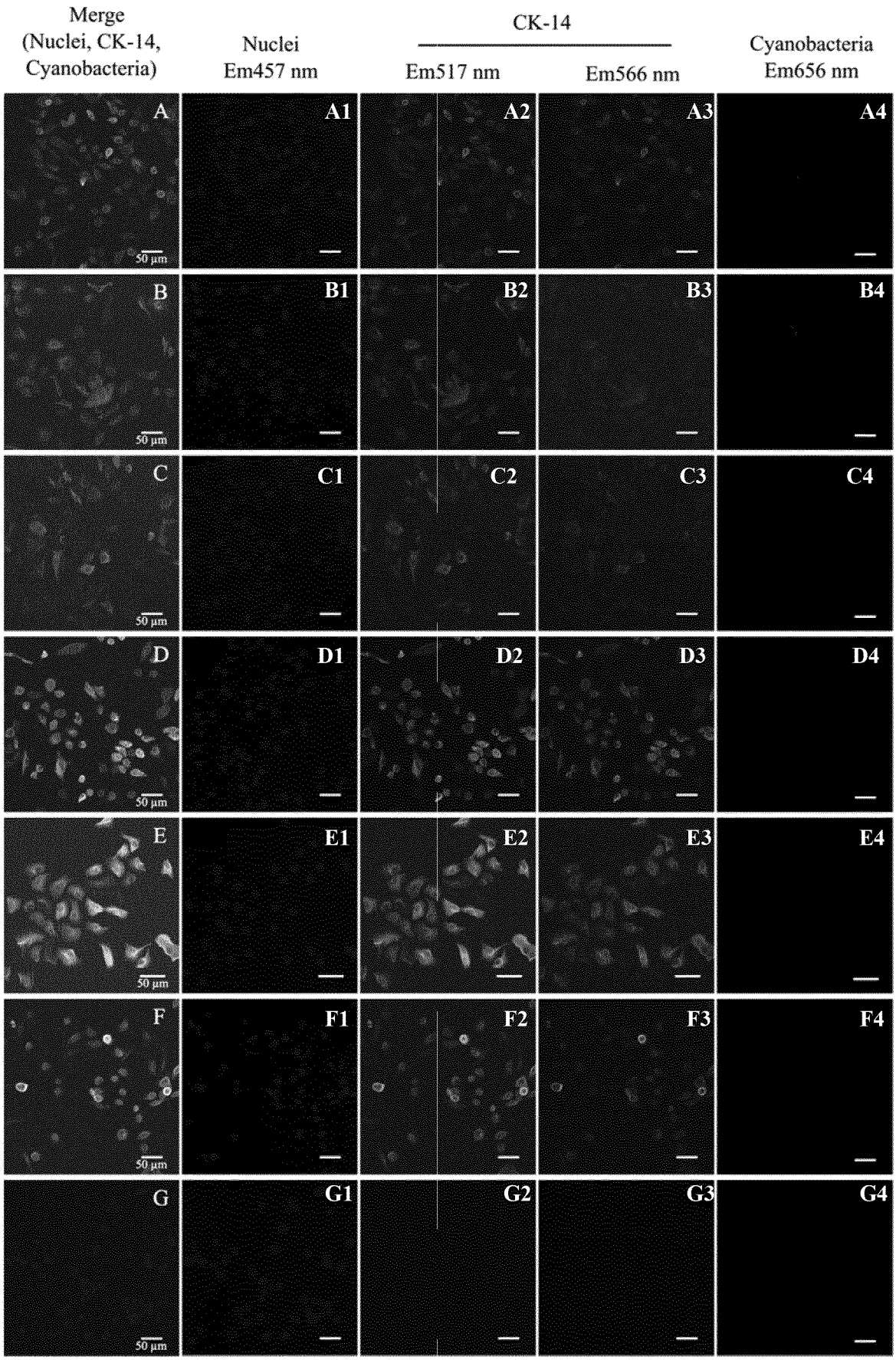

Merge
(Nuclei, CK-14,
Cyanobacteria)
Nuclei
Em457 nm
CK-14
Em517 nm
Em566 nm
Cyanobacteria
Em656 nm
A
A1
A2
A3
A4
B
B1
B2
B3
B4
C
C1
C2
C3
C4
D
D1
D2
D3
D4
E
E1
E2
E3
E4
F
F1
F2
F3
F4
G
G1
G2
G3
G4
50 µm

Fig. 19

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069322

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 A61K47/62 C12N5/10 G01N33/68 G01N33/74
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/057988 A1 (UNIV OF BREMEN [DE]) 28 March 2019 (2019-03-28) cited in the application	1-9, 11-15
A	the whole document section "summary of the invention" page 9 first paragraph examples 1, 2	10

X	CHRISTINA M. AGAPAKIS ET AL: "Towards a Synthetic Chloroplast", PLOS ONE, vol. 6, no. 4, 20 April 2011 (2011-04-20), page e18877, XP055694998, DOI: 10.1371/journal.pone.0018877 cited in the application	12,13
A	the whole document figure 1	1-11,14, 15

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2021	19/08/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jenkins, Gareth

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069322

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P. ARORA ET AL: "Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer", JOURNAL OF CELL SCIENCE, vol. 120, no. 6, 27 February 2007 (2007-02-27), pages 921-928, XP055748836, Cambridge ISSN: 0021-9533, DOI: 10.1242/jcs.03409	12,13
A	the whole document figure 3	1-11,15

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2021/069322

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2021/069322

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019057988 A1	28-03-2019	EP 3687579 A1	05-08-2020
		US 2020254110 A1	13-08-2020
		WO 2019057988 A1	28-03-2019