Targeted Hybrid Nanocarriers as a System Enhancing the Skin Structure

Agnieszka Lewińska 1,*, Marta Domżał-Kędzia 2, Kinga Kierul 3, Michał Bochynek 2, Dominika Pannert 3, Piotr Nowaczyk 4,5 and Marcin Łukaszewicz 2,*

1 Faculty of Chemistry, University of Wrocław, Joliot-Curie 14, 50-383 Wrocław, Poland
2 Department of Biotransformation, Faculty of Biotechnology, University of Wrocław, Joliot-Curie 14, 50-383 Wrocław, Poland; marta.domzal@uwr.edu.pl (M.D.-K.); michal.bochynek@uwr.edu.pl (M.B.)
3 InventionBio Sp. z o.o., Wojska Polskiego 65 st., 85-825 Bydgoszcz, Poland, kinga.kierul@inventionbio.pl (K.K.); dominika.pannert@inventionbio.pl (D.P.)
4 Faculty of Health Science, University of Opole, ul. Katowicka 68, 45-060 Opole, Poland; piotr.nowaczyk@uni.opole.pl
5 Dr. Nowaczyk Research and Innovation Center Sp. z o.o. Sp. K., ul. Żmigrodzka 81-83 lok. 205, 51-130 Wrocław, Poland
* Correspondence: agnieszka.lewinska@uwr.edu.pl (A.L.); marcin.lukaszewicz@uwr.edu.pl (M.L.)
Table SM1. The initial number of microbial cells in the inoculum (N) and the initial number of cells found in 1g of the product tested (N0).

| Test strain inoculum | The initial number of microorganisms in suspension cfu/mL (N) | The initial number of cells in 1g of the test product cfu/mL (N0) |
|----------------------|---------------------------------|---------------------------------|
| E. coli (ATCC 8739)  | 1.02 × 10^8                     | 1.02 × 10^6                     |
| S. aureus (ATCC 6538)| 9.3 × 10^7                      | 9.3 × 10^5                      |
| C. albicans (ATCC 10231)| 9.6 × 10^6               | 9.6 × 10^4                      |

Table SM2. Demonstration of neutralizer efficacy.

| Strain                      | Number of CFU (CFU/ml) A mixture of the neutralizer and diluent Nv (dilution 10^6) | A mixture of the neutralizer and sample Nv (dilution 10^6) | Nvn (dilution 10^6) |
|-----------------------------|----------------------------------------------------------------------------------|----------------------------------------------------------|---------------------|
| E. coli (ATCC 8739)         | 135                                                                               | 120                                                      | 135                 | 68                  |
| S. aureus (ATCC 6538)       | 187                                                                               | 201                                                      | 206                 | 103                 |
| C. albicans (ATCC 10231)    | 89                                                                                | 92                                                       | 83                  | 42                  |

For all tested strains, the effectiveness of the neutralizer was above 50%, i.e. the condition Nv ≥ 0.5 Nvn was met, and the number of microorganisms from the control sample (Nvn) was - as required - similar to the amount of the Nv strain.

Table SM3. Criteria for the evaluation of the preservation of cosmetic products.

| Bacteria | Yeast |
|----------|-------|
| Sample Timing (days) | 7 | 14 | 28 | 7 | 14 | 28 |
| Criteria A | A ≥3 and NI | ≥3 and NI | A ≥1 and NI2 | ≥1 and NI3 |
| Criteria B | A ≥3 | ≥3 and NI | A ≥1 | ≥1 |

A Not performed, 1 in this test, an acceptable range of deviation of 0.5 log is accepted, 2 NI – no increase in the count from the previous contact time, 3 Rx = 0 when lnN0 = lnNc (no increase from the initial count).

Table SM4. The number of viable cells of test microorganisms after a given contact time with nanoemulsion and levan nanocarriers.

| Preservative Symbol | N0 (CFU/ml) | A | AA | AB | AD | AE | F | G | K | L | N | R | W | Z |
|---------------------|-------------|---|----|----|----|----|---|---|---|---|----|---|---|---|---|
| NANOEMULSION        |             |   |    |    |    |    |   |   |   |   |    |   |   |   |   |
| Microbial strains   |             |   |    |    |    |    |   |   |   |   |    |   |   |   |   |
| E.coli              | 1.02 × 10^6 | 0 | NC | 0  | NC | 0  | 740| 1030| NC | 0  | NC | 1480|
| Microbial strains | E. coli | S. aureus | C. albicans |
|-------------------|---------|-----------|-------------|
| N14 (contact time) | 1.02 × 10⁶ | 9.3 × 10⁵ | 9.6 × 10⁴ |
| N28 (contact time) | 1.02 × 10⁹ | 9.3 × 10⁵ | 9.6 × 10⁴ |

NC not countable.

Table SM5. Log reduction values ($R_x = \log N_0 - \log N_x$).

| Preservative Symbol | A | AA | AB | AD | AE | F | G | K | L | N | R | W | Z |
|---------------------|---|----|----|----|----|---|---|---|---|---|---|---|---|
| NANOEMULSION        |   |    |    |    |    |   |   |   |   |   |   |   |   |
| Rx N14              | 6 | 2  | 6  | 2  | 6  | 6 | 3 | 3 | 2 | 6 | 6 | 2 | 3 |
| Rx N28              | 6 | 2  | 6  | 2  | 6  | 6 | 3 | 3 | 2 | 6 | 6 | 2 | 3 |
| LEVAN NANOCARRIER   |   |    |    |    |    |   |   |   |   |   |   |   |   |
| Rx N14              | - | 6  | -  | -  | -  | - | - | - | - | - | - | - | - |
| Rx N28              | - | 6  | -  | -  | -  | - | - | - | - | - | - | - | - |
Table SM6. Skin moisturization effect.

| Volunteer | Sex | First Measurement before Application (T0) | Second Measurement after Application (T1) Formulation C | Second Measurement after Application (T2) Formulation C1 | Difference (T1 − T0) | Difference (T2 − T0) |
|-----------|-----|------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|-----------------------|-----------------------|
| 1.        | F   | 35.5 ± 0.3                               | 37.6 ± 0.2                                            | 39.9                                                  | 2.1                   | 4.4                   |
| 2.        | F   | 35.9 ± 0.4                               | 38.2 ± 0.4                                            | 40.8 ± 0.6                                            | 2.3                   | 4.9                   |
| 3.        | F   | 36.7 ± 0.3                               | 37.9 ± 0.4                                            | 42.3 ± 0.6                                            | 1.2                   | 5.6                   |
| 4.        | F   | 37.3 ± 1.2                               | 40.0 ± 1.3                                            | 42.5 ± 0.8                                            | 2.7                   | 5.2                   |
| 5.        | F   | 36.0 ± 0.2                               | 39.2                                                  | 42.5 ± 0.2                                            | 3.2                   | 6.5                   |
| 6.        | F   | 36.7 ± 0.5                               | 38.7 ± 0.3                                            | 40.8 ± 1.1                                            | 2.0                   | 4.1                   |
| 7.        | F   | 37.4                                                  | 39.6 ± 0.7                                            | 42.4                                                  | 2.2                   | 5.0                   |
| 8.        | F   | 36.3 ± 1.1                               | 38.4                                                  | 42.5 ± 1.0                                            | 2.1                   | 6.2                   |
| 9.        | F   | 35.8 ± 0.7                               | 38.8 ± 0.2                                            | 43.0                                                  | 3.0                   | 7.2                   |
| 10.       | F   | 34.3 ± 1.6                               | 38.0 ± 0.3                                            | 38.8 ± 2.1                                            | 3.7                   | 4.5                   |
| Mean value|     | 36.2                                    | 38.6                                                  | 41.6                                                  | 2.4                   | 5.4                   |

Assumption: an increase in the measurement value over time means greater skin moisturization.

Table SM7. Influence of skin firmness/elasticity.

| Volunteer | Sex | First Measurement before Application (T0) | Second Measurement after Application (T1) Formulation C | Second Measurement after Application (T2) Formulation C1 | Difference (T1 − T0) | Difference (T2 − T0) |
|-----------|-----|------------------------------------------|--------------------------------------------------------|--------------------------------------------------------|-----------------------|-----------------------|
| 1.        | F   | 36.0 ± 0.2                               | 36.6 ± 0.5                                            | 36.9 ± 0.6                                            | 0.6                   | 0.9                   |
| 2.        | F   | 35.4 ± 0.4                               | 37.8 ± 0.7                                            | 37.8 ± 0.1                                            | 2.4                   | 2.4                   |
| 3.        | F   | 35.6                                                  | 37.0 ± 0.5                                            | 37.7 ± 0.1                                            | 1.4                   | 2.1                   |
| 4.        | F   | 36.2 ± 0.9                               | 37.0                                                  | 36.9 ± 0.5                                            | 0.8                   | 0.7                   |
| 5.        | F   | 35.4                                                  | 36.9 ± 2.0                                            | 36.9                                                  | 1.5                   | 1.5                   |
| 6.        | F   | 35.9 ± 0.1                               | 38.0 ± 0.2                                            | 38.7 ± 0.8                                            | 2.1                   | 2.8                   |
| 7.        | F   | 35.5 ± 1.1                               | 37.8                                                  | 38.3 ± 1.3                                            | 2.3                   | 2.8                   |
| 8.        | F   | 36.7 ± 0.1                               | 38.1 ± 0.8                                            | 38.7 ± 0.1                                            | 1.4                   | 2.0                   |
| 9.        | F   | 36.0 ± 1.3                               | 39.9 ± 0.3                                            | 39.7 ± 2.9                                            | 3.9                   | 3.7                   |
| 10.       | F   | 37.3 ± 0.5                               | 39.0 ± 0.7                                            | 40.0 ± 0.9                                            | 1.7                   | 2.7                   |
| Mean value|     | 36.0                                    | 37.8                                                  | 38.2                                                  | 1.8                   | 2.2                   |

Assumption: an increase in the measurement value over time means greater skin firmness/elasticity.
### Table SM8. Skin smoothening effect.

| Volunteer | Sex | Skin Firmness (px) before Application (T0) | Skin Firmness (px) after Application(T1) | Skin Firmness (px) Difference (T1 − T0) | Skin Firmness (px) Difference (T2 − T0) |
|-----------|-----|--------------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| 1.        | F   | 11.0                                       | 10.0 ± 1.0                               | −1.0                                     | −1.0                                     |
| 2.        | F   | 12.0 ± 1.2                                 | 11.0                                     | −1.0                                     | −2.0                                     |
| 3.        | F   | 12.0 ± 1.0                                 | 12.0 ± 1.5                               | 0                                        | 0                                        |
| 4.        | F   | 12.0                                       | 11.0 ± 0.6                               | −1.0                                     | −1.0                                     |
| 5.        | F   | 13.0 ± 2.3                                 | 12.0                                     | −1.0                                     | −2.0                                     |
| 6.        | F   | 11.0                                       | 10.0 ± 1.5                               | −1.0                                     | −1.0                                     |
| 7.        | F   | 12.0 ± 2.0                                 | 12.0                                     | 0                                        | 0                                        |
| 8.        | F   | 12.0 ± 1.2                                 | 12.0 ± 1.0                               | 0                                        | 0                                        |
| 9.        | F   | 13.0                                       | 12.0                                     | −1.0                                     | −1.0                                     |
| 10.       | F   | 11.0 ± 1.7                                 | 10.0 ± 1.7                               | −1.0                                     | −1.0                                     |
| Mean value|     | 11.9                                       | 11.2                                     | −0.7                                     | −0.9                                     |

Assumption: a decrease in the measurement value over time means an increase in the smoothness of the epidermis.

### Table SM9. Reduction of wrinkles. Base cream.

| Volunteer | Sex | The Volume of Wrinkles (px3) Before Application (T0) | The Volume of Wrinkles (px3) After Application (T1) | The Volume of Wrinkles Difference (T1 − T0) | Wrinkles Surface (px2) Before Application (T0) | Wrinkles Surface (px2) After Application (T1) | Wrinkles Surface Difference (T1 − T0) | The Depth of Wrinkles (px) (Before Application) (T0) | The Depth of Wrinkles (px) (After Application) (T1) | The Depth of Wrinkles Difference (T1 − T0) |
|-----------|-----|------------------------------------------------------|-----------------------------------------------------|--------------------------------------------|-----------------------------------------------|--------------------------------------------|----------------------------------------|---------------------------------------------|-----------------------------------------------|---------------------------------------------|
| 1.        | F   | 97452                                                | 96001                                               | −1451                                      | 7849                                          | 7701                                       | −148                                   | 12.0                                        | 11.0                                          | −1.0                                        |
| 2.        | F   | 97225                                                | 95293                                               | −1932                                      | 7855                                          | 7628                                       | −227                                   | 12.0                                        | 12.0                                          | 0                                            |
| 3.        | F   | 99567                                                | 97449                                               | −2118                                      | 7931                                          | 7703                                       | −228                                   | 11.0                                        | 11.0                                          | −1.0                                        |
| 4.        | F   | 98473                                                | 96332                                               | −2141                                      | 7820                                          | 7519                                       | −301                                   | 12.0                                        | 12.0                                          | −1.0                                        |
| 5.        | F   | 96201                                                | 95141                                               | −1060                                      | 7744                                          | 7611                                       | −133                                   | 12.0                                        | 12.0                                          | 0                                            |
| 6.        | F   | 97662                                                | 95214                                               | −2448                                      | 7900                                          | 7716                                       | −184                                   | 13.0                                        | 12.0                                          | −1.0                                        |
| 7.        | F   | 96778                                                | 95002                                               | −1776                                      | 7846                                          | 7594                                       | −252                                   | 12.0                                        | 12.0                                          | 0                                            |
| 8.        | F   | 100258                                               | 97391                                               | −2867                                      | 7922                                          | 7659                                       | −263                                   | 11.0                                        | 11.0                                          | 0                                            |
| 9.        | F   | 99364                                                | 96888                                               | −2476                                      | 7833                                          | 7600                                       | −233                                   | 11.0                                        | 10.0                                          | −1.0                                        |
| 10.       | F   | 97555                                                | 95719                                               | −1836                                      | 7749                                          | 7518                                       | −231                                   | 12.0                                        | 11.0                                          | −1.0                                        |
| Mean value|     | 98054                                                | 96043                                               | −2011                                      | 7845                                          | 7625                                       | −220                                   | 11.8                                        | 11.2                                          | −0.6                                        |
Table SM10. Reduction of wrinkles. Formulations with nanosystems.

| Volunteer | Sex | The Volume of Wrinkles (px³) Before Application (T0) | The Volume of Wrinkles (px³) After Application (T1) | The Volume of Wrinkles Difference (T1 − T0) | Wrinkles Surface (px²) Before Application (T0) | Wrinkles Surface (px²) After Application (T1) | Wrinkles Surface Difference (T1 − T0) | The Depth of Wrinkles (px) Before Application (T0) | The Depth of Wrinkles (px) After Application (T1) | The Depth of Wrinkles Difference (T1 − T0) |
|-----------|-----|-----------------------------------------------------|-----------------------------------------------------|------------------------------------------|----------------------------------------------|----------------------------------------------|------------------------------------------|-----------------------------------------------|-----------------------------------------------|------------------------------------------|
| 1.        | F   | 97420                                               | 95752                                               | −1668                                    | 7856                                         | 7677                                         | −179                                      | 12.0                                          | 12.0                                          | 0                                        |
| 2.        | F   | 97239                                               | 95001                                               | −2238                                    | 7870                                         | 7601                                         | −269                                      | 12.0                                          | 11.0                                          | −1.0                                    |
| 3.        | F   | 99555                                               | 97001                                               | −2554                                    | 7946                                         | 7640                                         | −306                                      | 11.0                                          | 10.0                                          | −1.0                                    |
| 4.        | F   | 98443                                               | 95743                                               | −2700                                    | 7805                                         | 7479                                         | −326                                      | 11.0                                          | 10.0                                          | −1.0                                    |
| 5.        | F   | 96210                                               | 95012                                               | −1198                                    | 7713                                         | 7580                                         | −133                                      | 12.0                                          | 12.0                                          | 0                                        |
| 6.        | F   | 97678                                               | 95123                                               | −2555                                    | 7909                                         | 7697                                         | −212                                      | 12.0                                          | 11.0                                          | −1.0                                    |
| 7.        | F   | 96760                                               | 94706                                               | −2054                                    | 7855                                         | 7590                                         | −265                                      | 12.0                                          | 11.0                                          | −1.0                                    |
| 8.        | F   | 100240                                              | 97111                                               | −3129                                    | 7914                                         | 7663                                         | −251                                      | 11.0                                          | 11.0                                          | 0                                        |
| 9.        | F   | 99347                                               | 96890                                               | −2457                                    | 7820                                         | 7640                                         | −180                                      | 11.0                                          | 11.0                                          | 0                                        |
| 10.       | F   | 97559                                               | 95640                                               | −1919                                    | 7779                                         | 7407                                         | −372                                      | 12.0                                          | 11.0                                          | −1.0                                    |
| Mean value|     | 98045                                               | 95798                                               | −2247                                    | 7847                                         | 7597                                         | −250                                      | 11.6                                          | 11.0                                          | −0.6                                    |

Table SM11. Microbial limits for cosmetic products according to EN ISO 17516: 2014.

| Types of Microorganisms | Other Products |
|-------------------------|----------------|
| Total Aerobic Mesophilic Microorganisms (Bacteria plus yeast and mold) | ≤ 1 × 10³ CFU per g or ml |
| *Escherichia coli* | Absence in 1 g or 1 ml |
| *Pseudomonas aeruginosa* | Absence in 1 g or 1 ml |
| *Staphylococcus aureus* | Absence in 1 g or 1 ml |
| *Candida albicans* | Absence in 1 g or 1 ml |

Table SM12. Media used to assess the effectiveness of product preservation.

| Medium | Composition |
|--------|-------------|
| Trypsic Soy Agar (TSA) | Pancreatic digest of casein 15 g/L, papain digest of soybean meal 5 g/L, sodium chloride 5 g/L, agar 15 g/L; Final pH 7.3 ± 0.2 at 25 °C |
| D/E Neut Broth | Dextrose 10 g/L, Lecithin 7 g/L, Sodium Thioglycollate 6 g/L, Pancreatic Digest of Casein 5 g/L, Tween® 80 5 g/L, Yeast Extract 2.5 g/L, Sodium Bisulfite 2.5 g/L |
| Sabouraud Dextrose Agar (SDA) | Glucose 40 g/L, a peptic digest of animal tissue 5 g/L, a pancreatic digest of casein 5 g/L, agar 15 g/L; Sodium Thioglycollate 1 g/L, Monopotassium Phosphate 0.1 g/L, Bromcresol Purple 0.02 g/L; Final pH 7.6 ± 0.3 at 25 °C |

SM 1. Microbiological analysis
Preserved nanosystems were subjected to microbiological tests. Namely, 1 g of preserved nanosystem was 10 times diluted with neutralizing broth DE (Dey/Engley broth, Oxoid) and incubated at room temperature for 30 min. Such prepared dilution was pipetted on a Petri dish and 15-20 ml of melted agar was poured, the temperature of agar not exceeding 48 °C. Petri dishes were gently rotated to disperse the sample. For detection of mesophilic bacteria, Tryptic Soy Agar (TSA, Oxoid) was used. For detection of yeast and molds Sabouraud Dextrose Agar (SDA, Oxoid) was used. Plates were incubated for 72 h at 25 ± 2.5 °C.

SM 2. Evaluation of microbial protection: criteria and experimental conditions

Preservation efficiency test (Challenge test) was performed according to the method described in normative document ISO 11930. Nanocarrier systems have been subjected to stress tests in order to assess the effectiveness of preservation (EN ISO 11930: 2012 Cosmetics - Microbiology - Evaluation of the antimicrobial protection of a cosmetic product (ISO 11930: 2012)). Obtained results were assessed based on criteria in Table SM3.

Challenge test was conducted using two bacterial strains Escherichia coli (ATCC 8739), Pseudomonas aeruginosa (ATCC 9027), and yeast strain Candida albicans (ATCC 10231). Bacterial suspensions were obtained in a way that reaches $1 \times 10^5 – 1 \times 10^6$ of Colony Forming Units (CFU) for bacteria and $1 \times 10^4 – 1 \times 10^5$ CFU for yeast in tested formulations. All microbial media used during the test are listed in Table SM 12.

Bacteria were incubated at 30 - 35 °C for 48 – 72 hours.

SM. 3 Method used for inoculum enumeration

For the enumeration of viable microorganisms in the inoculum, the pour plate method was used: 1 ml of the appropriate decimal dilution was applied to the Petri dish and subsequently 15 - 20 ml of melted agar medium was poured.

SM. 4 Preparation of bacterial suspensions and their incorporation into the tested formulations

To prepare the infecting inoculum of the test strain, the loopful of bacterial cells grown on an agar plate was transferred to a sterile tube containing 5 ml of the diluent D/E. Bacterial suspensions were set to contain $1 \times 10^7 – 1 \times 10^8$ CFU, for yeast $1 \times 10^6 – 1 \times 10^7$ CFU. The exact number of cells in the infecting suspensions (N) has been determined, by the preparation of subsequent decimal dilutions.

On this basis, the initial number of cells in 1 g of the tested product ($N_0$) was calculated. The results are presented in Table SM 1.

SM. 5. Determination of the effectiveness of a preservative

To validate whether the applied diluent can neutralize the preservative without inhibiting the growth of microorganisms, the effectiveness of the neutralizer was determined. For this purpose, the number of microorganisms determined in the microbial suspension was incubated in the presence and absence of the test product and the neutralizer. The applied neutralizer is considered efficient when the recovery of microorganisms in the presence of the tested product is at least 50% concerning the number of microorganisms not treated with the tested product. The results of determining the effectiveness of the neutralizer are presented in Table SM 2.

SM. 6 Determination of the preservation efficacy
The number of viable microbial cells was assessed for all tested preservation systems for both, nanoemulsion and levan nanoparticles. Tests were prepared for samples at 14 and 28 days of contact, using the method mentioned in SM1. The test results are presented in Tables SM. 4 and SM. 5.

The study determined the number of cells of the test microorganisms after the specified contact times of the preparation with the inoculated test microorganisms. The contact times were 14 days and 28 days for bacteria and C. albicans, and 14 and 28 days for A. brasiliensis, respectively. To determine the number of live cells in 1 g of the tested product, inoculation was performed using the flooding method, using the diluent specified in PN-EN ISO 11930: 2012 standard. Before sowing in Petri dishes, the product was left in the diluent for 30 ± 15 minutes at room temperature. The test results are presented in Tables SM. 4 and SM. 5.

**Figure SM1.** Images of vascular lesions and discoloration for the tested skin.