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Full paper

Hyaluronan-Based Nanohydrogels for Targeting Intracellular *S. aureus* in Human Keratinocytes

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1 **Abstract**

2 *Staphylococcus aureus* is one of the most significant human pathogens that is frequently isolated in
3 a wide range of superficial and systemic infections. The ability of *S. aureus* to invade and survive
4 within host cells such as keratinocytes and host immune cells has been increasingly recognised as a
5 potential factor in persistent infections and treatment failures. The incorporation of antibiotics into
6 hyaluronan-cholesterol nanohydrogels (NHs) represents a novel paradigm in the delivery of
7 therapeutic agents against intracellular bacteria. The work presented herein shows that NHs quickly
8 enter human keratinocytes and accumulate into lysosomes. When used for targeting intracellular *S.*
9 *aureus* the antimicrobial activity of loaded levofloxacin (LVF) is enhanced, possibly changing the
10 antibiotic intracellular fate from cytosol to lysosome. Indeed, gentamicin (GM), an antibiotic that
11 predominantly accumulates in lysosomes, showed significant and equal antibacterial activity when
12 entrapped into NHs. These results strongly suggest that lysosomal formulations may display
13 preferential activity towards intracellular *S. aureus*, opening new avenues for the use of HA-based
14 NHs for treatment of such skin infections.

15

16 **1. Introduction**

17 The opportunistic pathogen *S. aureus* is a prevalent commensal organism and a significant
18 mammalian pathogen, frequently associated with a wide range of clinical infections that include
19 skin, soft tissues and device related infections and bacteraemia^[1]. Whilst *S. aureus* is not
20 traditionally considered as an intracellular pathogen, there is increasing evidence which suggests
21 that this microorganism can not only invade and persist in a range of cell types^[2], but also that this
22 adaptation may offer protection from the immune response and be a factor in treatment failure due
23 to the inability of the antibiotic/antimicrobial agent to target intracellular microorganisms. This
24 advantageous facet of intracellular adaption is evident in intracellular pathogens such as
25 *Mycobacteriae* which can survive and replicate within macrophages by resisting lysosomal delivery

26 by residing in early phase endosomal compartments^[3], whilst organisms such as *Salmonellae* and
27 *Brucellae* survive by preventing vacuole-lysosome fusion^[4] and pathogens such as *Shigella spp.*
28 *Listeria spp* and *Rickettsiae* are able to escape from phagosomes and survive into the cytosol^[5]. To
29 survive and disseminate intracellularly *S. aureus* has developed some specific adaptations; one is to
30 resist the fusion of phagosomes with lysosomes and to multiply within the phagolysosomes of
31 macrophages^[6], whilst others, for example thought such as FnBP-dependent and -independent
32 pathways have been shown to facilitate *S. aureus* internalization and survival within human
33 keratinocytes^[2b, 7]. Once internalized, it has been proposed that this offers protection from humoral
34 immune responses and from the action of several antibiotics^[8].

35 When treating an intracellular infection, a suitable antibiotic should be chosen in order to ensure
36 drug concentration above the minimum inhibitory concentration (MIC) and in the same intracellular
37 location as the target microorganism. Depending on their physico-chemical properties, antibiotics
38 accumulate in various cell compartments at different concentrations; typically, weak bases tend to
39 accumulate in membrane-bound acidic compartments, whereas weak acids are excluded from
40 those sites^[9]. Specifically, aminoglycosides^[10] and macrolides^[11] predominantly accumulate in
41 lysosomes, whereas quinolones^[8a, 12] accumulate in the cytosol. The β -lactams group of
42 antibiotics^[8b] have been shown to accumulate at low level within the cells (predominantly in the
43 cytosol) likely due to their acidic character. Despite the ability of antibiotics to cross cell membrane,
44 their intracellular efficacy can be poor due to: I) low intracellular concentrations below the minimum
45 inhibitory value; II) intracellular environment (e.g. acidic pH) that may affect the antibiotic activity;
46 III) antibiotic accumulation in subcellular compartments that are different from those in which
47 pathogens persist. These limitations could potentially be overcome by utilizing suitable nano-
48 carriers which can enhance the intracellular uptake of antibiotics^[13] and facilitate the subcellular
49 targeting^[14]. Such nano-carriers can be customized in order to target lysosomes or to escape the
50 endosome, being released into the cytosol. Indeed, carriers made of polycations can provide a

51 'proton sponge effect'^[15] or such carriers can be conjugated to membrane-degrading/destabilizing
52 peptides in order to break down the endosome membrane, allowing the 'cargo' release into the
53 cytosol^[16]. In this respect, a number of strategies have been studied for achieving drug delivery
54 systems that can target specific cellular compartments^[14a, 17]. Hyaluronan (HA), a generally non-toxic
55 and non-sulphated glycosaminoglycan, is a natural component of human skin and is predominantly
56 employed in the cosmetics industry as a dermal filler ^[18] or skincare agent or in
57 viscosupplementation^[18b]. A major receptor for HA is CD44, a cell-surface glycoprotein highly
58 expressed on the surface of keratinocytes^[19] and activated macrophages^[20]. After binding to CD44,
59 HA is taken up by cells and delivered to the lysosomes^[21]. CD44 appears to be important in the
60 invasion, survival and persistence of a range of microorganisms within host cells^[22]. For example,
61 *Group A Streptococcus* (GAS) has been shown to attach to epithelial cells through its HA-rich
62 polysaccharide capsule which is reported to mediate attachment to CD44 on pharyngeal and
63 epidermal keratinocytes^[23]. Moreover, CD44 was identified as a widely distributed receptor in the
64 epithelial tissues where colonization and infection occur and represented the major cellular
65 receptor for the GAS entry into primary mouse keratinocytes^[22b]. CD44 has also been implicated in
66 the cellular internalization of other pathogens such as *Mycobacterium tuberculosis* in
67 macrophages^[22a] and for *Shigella spp* in epithelial cells^[22d]. Consequently, the application of HA-
68 based nano-carriers may be particularly suitable for targeting intracellular pathogens as: I) a number
69 of host cells (e.g. keratinocytes and macrophages) highly express CD44 and easily internalize HA; II)
70 HA can enter cells through the receptor (CD44) that is also employed by such pathogens; III) like
71 other nanoparticles, HA nano-carriers can be engineered in order to target lysosomes or other
72 cellular compartments^[24]; IV) in the cellular micro-environment the HA nano-carriers may be
73 cleaved by hyaluronidases (HA_{ase}) that are produced by several bacteria such as *Staphylococcus spp.*
74 and *Streptococcus spp.*^[25], facilitating the release of the drug *in situ*. Moreover, the scission of HA
75 can also occur in the presence of host enzymes, acidic pH and free radicals^[26], assuring the drug

76 release within the cells, thus guaranteeing the efficacy of the targeted therapy also against
77 microorganisms that typically do not produce HA_{ase} such as *P. aeruginosa*^[27] and *M. Tuberculosis*^[28].
78 The data presented herein show the novel application of self-assembling HA-based NHs^[29] for
79 enhancing the intracellular uptake of antibiotics into human keratinocytes and targeting to
80 intracellular *S. aureus*. The efficacy of GM or LVF versus -loaded NHs (NH/GM or NH/LVF,
81 respectively) was explored. These two antibiotics were selected as they have different intracellular
82 pathways: GM is a lysosomal drug^[10], whilst LVF is a cytosolic one^[8a]. GM is typically used in topical
83 preparations and can be employed to treat skin infections, whilst LVF remains among the most
84 active oral antimicrobials.

85

86 **2. Results and Discussion**

87 **2.1. Development and characterization of NH/GM and NH/LVF**

88 In order to generate NHs, HA carboxyl groups were functionalized with a small hydrophobic
89 molecule, cholesterol (CH)^[29]. In a previous work, the *soft* nature, the swelling ability, the spherical
90 shape and size of HA-CH NHs in both dry and aqueous environments (by using Atomic Force
91 Microscopy and Transmission Electron Microscopy techniques) were showed^[30]. These
92 nanoparticles evidenced the ability to absorb a high amount of water and to swell in aqueous media,
93 thus showing the typical properties of NHs^[31, 32]. As HA chains are chemically modified with a
94 hydrophobic moiety (CH), the resulting amphiphilic polymer is able to self-assemble in aqueous
95 environment and spontaneously forms physically cross-linked tri-dimensional networks which are
96 characterized by internal hydrophobic domains and hydrophilic outer chains. Depending on the
97 degree of functionalization and on the polymer molecular weight, these aggregates may form
98 regular nano-structures, after suitable treatments such as sonication or autoclaving.

99 In this respect, it was already shown that autoclaving (121°C, 20 min) represents a fast and
100 reproducible one-pot method for directly achieving sterile and drug-loaded NHs^[33]. In this way,

101 both sterile NH/GM and NH/LVF were obtained after autoclaving (**Figures 1 and 2**). Nano-systems
102 were purified from the free antibiotics using a size exclusion chromatography (SEC) column; drug
103 loading (DL, %) and loading efficiency (LE, %) were studied with an ultra-performance liquid
104 chromatography (UPLC) coupled with a mass-spectrometer (NH/GM) and with a UV-Vis
105 spectrometer (NH/LVF). The amount of entrapped drug is reported in **Table 1** and shows that GM
106 loading was higher than that of LVF; this may be due to the amino groups of the GM sugar rings,
107 which exhibit a net positive charge under physiological conditions (pKa values of GM amine groups
108 range from 5.6 to 9.5), establishing strong electrostatic interactions with the negatively charged HA
109 chains. In contrast, LVF exists as a zwitterion at pH 6.0-7.5, leading to weaker interactions with NHs
110 and, thus, lower encapsulation values. NH/GM and NH/LVF were characterized in terms of mean
111 diameter, PDI and ζ -pot (as summarized in Figure 1A, B, C and 2A, B, C), showing average sizes of ~
112 250 nm (NH/GM) and ~ 350 nm (NH/LVF). It was necessary the use of different solvents for the
113 formulation of NH/GM and NH/LVF; pH values lower than 7 were not suitable for the formation of NH/GM
114 as a precipitate was found after the autoclaving process (possible due to the strong electrostatic interactions
115 occurring between GM and HA, which prevent the NHs formation by self-assembling). Whilst, less LVF was
116 loaded into NHs at neutral pH compared to that loaded in double-distilled water (pH=5). This result can be
117 ascribed to the zwitterionic nature of LVF. Therefore, to maximize the concentration of antibiotics loaded in
118 the NHs a different solvent for each antibiotic was used. As expected, an increase in the amount of
119 loaded GM led to a decrease of the ζ -pot net value of NHs (due to the positively charged GM)
120 compared to that of the unloaded NHs. Indeed, the GM DL% of $40 \pm 1\%$, $35.1 \pm 2.8\%$ and $30.7 \pm 2\%$
121 (w/w, %) led to a ζ -pot change of 49%, 24% and 20%, respectively, compared to that of free NHs,
122 evidencing a GM concentration-dependent effect. Loaded LVF affected NHs ζ -pot to a lesser extent.
123 Moreover, both NH/GM and NH/LVF stored at -20°C or freeze-dried as solid powders, retained their
124 mean diameter and PDI when defrosted and re-hydrated. For preserving the starting NH/GM and
125 NH/LVF properties, it was necessary to add dextrose before freeze-drying (Figure 1A and B). The

126 NHs freeze-dried powder ensures the long-term storage of the nano-formulations, as the stability
127 of the aqueous suspensions of NHs and the loaded drugs is rather limited. The antimicrobial activity
128 of NH/LVF freeze-dried powder was already studied against intracellular *S. aureus* and *P.*
129 *aeruginosa*^[27], evidencing the storage method is able to retain the whole activity of the nano-
130 formulation. Furthermore, the NHs freeze-dried powder could be mixed with appropriate materials
131 (e.g. carbopol), at suitable concentrations, in order to obtain formulations for topical
132 administrations. Figs. 1 and 2 also show that liquid samples can be frozen, retaining their size and
133 PDI and assuring a long-term storage without the addition of any additive.

134 Among the prepared samples, NH/GM and NH/LVF with the starting 1:1 wt. ratio (formulations
135 written in bold in Table 1) showed the best properties and loading efficiency, therefore they were
136 selected for subsequent experiments. To assess the stability of loaded NHs in aqueous
137 environments, drug release studies (Figure 1D and 2D) were performed in double-distilled water
138 (pH ~ 5 similar to that of the human skin). Results showed that only 10% (% w/w) of loaded GM was
139 released from NHs in 24 h, evidencing the high stability of the nano-formulation. Longer time points
140 were also checked over one week (data not shown), but no changes were observed in the release
141 profile. This behaviour can be explained by taking into account that the electrostatic interactions
142 between the negatively charged NHs and the positively charged GM are very strong at low ionic
143 strength. However, some degradation mechanisms should occur in '*in vitro*' or '*in vivo*' (e.g. specific
144 enzymes such as HA_{ase} and esterase, acidic pH and free radicals), enhancing the GM release from
145 the NHs. In contrast, 48% (% w/w) of loaded LVF was released over 24 h in the same conditions; this
146 result can be ascribed to weaker interactions within the NHs.

147

148 **2.2. Cell viability of HaCaT**

149 To determine the effects of antibiotics-loaded NHs on cell viability, HaCaT cells were incubated with
150 NH/GM or NH/LVF or their antibiotic-free controls over 48 h. Both MTT (**Figure 3A and C**) and trypan-

151 blue (Figure 3B and D) assays showed that NHs preparations were not toxic to HaCaT, as neither
152 HaCaT metabolism nor vitality was significantly affected by any of the tested concentrations after
153 48 h (Figure 3).

154
155

156 **2.3. Antibacterial activity of NH/GM and NH/LVF against extracellular and intracellular** 157 ***S.aureus***

158 To ensure the NHs encapsulation do not enhance or inhibit antimicrobial activity, the MIC and the
159 minimum bactericidal concentration (MBC) of NHs alongside free GM and LVF, NH/GM and NH/LVF
160 were determined; **figure 4A** shows the mean MIC and MBC values. Free NHs did not show any
161 antimicrobial activity and incorporation of GM or LVF into NHs did not affect their antibacterial
162 activity against extracellular *S. aureus*. The efficacy of the nano-formulations against intracellular *S.*
163 *aureus* was then assessed by enumerating viable intracellular bacteria from infected HaCaT treated
164 with NH/GM, NH/LVF or their controls, in conditions which did not affect the viability of HaCaT cells,
165 by using the following concentrations: c = 46.8 µg/mL (GM) and c = 117 µg/mL (NHs) for the NH/GM
166 system and at c = 35.1 µg/mL (LVF) and c = 307 µg/mL (NHs). Antibiotic concentrations tested were
167 3 fold greater than the MIC/MBC to ensure eradication of viable bacterial cells. After 2 h of
168 incubation, NH/GM and NH/LVF and their controls were not effective against intracellular *S. aureus*
169 (Figure 4B). After 5 h of incubation both free GM and NH/GM showed significant antimicrobial
170 activity against the intracellular *S. aureus* ($p < 0.05$); however, when compared to free GM, NHs did
171 not enhance GM activity intracellularly. In contrast, LVF activity was highly enhanced by NHs
172 ($p < 0.005$) when compared to that of free LVF (Figure 4B).

173

174 **2.4. Uptake and intracellular fate of HA-CH NHs in HaCaT**

175 Rhodamine B-isothiocyanate (rhod) dye was covalently linked to the hydroxyl groups of NHs and the
176 obtained fluorescent NHs were employed for studying their binding/internalization kinetics and

177 intracellular fate into HaCaT by flow cytometry and fluorescence microscopy. Flow cytometry
178 analysis evidenced that HaCaT incubated for 30 min with rhod-NHs showed a significant increase in
179 fluorescence intensity, indicating a quick binding/uptake of NHs within the cells. HaCaT fluorescence
180 steadily increased over the time and 4 h represented the time point at which the highest signal was
181 detected, followed by a plateau up to 24 h (**Figure 5A**). Moreover, HaCaT cells showed increased
182 fluorescence in a dose-dependent manner with rhod-NHs concentrations up to 100 µg/mL after 4 h
183 of incubation (Figure 5B).

184 ApoTome analysis showed NHs located into vesicle-like structures, those with a diameter of
185 approximately 0.3 µm close to the plasma membrane and those in larger vesicles with diameter up
186 to 1.5 µm close to the nucleus, suggesting an intracellular location of NHs (**Figure 6**). Interestingly,
187 Tammi et al. (2001) had a similar outcome using free HA in rat epidermal keratinocytes ^[19a]. To
188 determine the intracellular fate of NHs and explore the possibility of their co-localization with
189 lysosomes, HaCaT were then incubated up to 24 h with the nano-system and stained with 'Lyso-
190 tracker Green DND-26' (**Figure 7**). At 1 h a limited number of NHs was located in lysosomes;
191 however, from 4 to 24 h a strong co-localization occurred (*****P** value < 0.005) compared to that at
192 1 h, showing the highest Pearson correlation coefficient value ^[34] (0.94) at 4 h. NHs in lysosomes
193 were still evident up to 24 h, although co-localization was weaker than that found at 4 h (~ 0.7)
194 (Figure 7); no significant differences were found in the time points ranging from 6 to 24 h.

195 Since a previous work showed that LVF predominantly accumulates in the cytosol, it is plausible that
196 NHs are able to change the intracellular fate of LVF (from cytosol to lysosome), delivering the
197 antibiotic to the intracellular location of *S. aureus* (**Figure 8**), thus enhancing its intracellular
198 antimicrobial activity (Figure 4B). On the other hand, GM, an antibiotic that predominantly
199 accumulates in lysosomes, showed a significant antibacterial activity without the presence of NHs.
200 These results strongly suggest that therapeutics that are able to accumulate in lysosomes might be
201 more effective against intracellular *S. aureus* compared with those that accumulate in other cell

202 compartments (e.g. cytosol), evidencing that: I) sub-cellular targeting could be necessary for
203 eradicating the intracellular pathogen; II) lysosomal nanoparticles (e.g. HA-CH NHs) might be
204 suitable for enhancing the antibacterial activity of such antibiotics against the intracellular *S. aureus*
205 in human keratinocytes.

206

207 **3. Conclusion**

208 We have shown that self-assembled NHs can be loaded with GM or LVF antibiotics by means a fast
209 one-step sterile cycle using an autoclave (121°C for 20 min). NH/GM and NH/LVF show mean
210 diameters of approximately 250 and 350 nm, respectively; both nano-formulations do not affect
211 viability of HaCaT cells at all tested concentrations over 48 h and display the same MIC and MBC
212 values as free antibiotics against extracellular *S. aureus*. However, intracellularly, the antibacterial
213 activity of LVF was highly enhanced by NHs. As we have demonstrated that NHs co-localize with
214 lysosomes of HaCaT cells and it is known that free LVF predominantly accumulates in the cytosol,
215 these results strongly suggest that NHs may be able to change the intracellular fate of LVF from
216 cytosol to lysosome, thereby targeting intracellular *S. aureus*. Indeed, GM, an antibiotic that
217 predominantly accumulates in lysosomes, shows significant intracellular activity without the
218 employment of NHs. To conclude, this research demonstrates that lysosomal formulations may be
219 more effective against *S. aureus* in keratinocytes compared with formulations that accumulate in
220 other cell compartments (e.g. cytosol), opening new avenues for HA-based NHs treatments of
221 persistent *S. aureus* skin infections.

222

223 **4. Experimental section**

224 *Materials:* Hyaluronan tetrabutylammonium salt (HA-TBA⁺, M_w = 2.2 x 10⁵) was purchased from
225 Contipro (Dolní Dobrouč, Czech Republic). Cholesterol (CH), 4-bromobutyric acid, N-methyl-2-
226 pyrrolidone (NMP), N-(3-dimethylaminopropyl)-N'-(ethylcarbodiimide hydrochloride) (EDC·HCl),
227 gentamicin sulfate (GM), levofloxacin (LVF), 4-(dimethylamino)pyridine (DMAP), phosphate

228 buffered saline tablets, dextrose, dimethyl sulfoxide ACS reagent $\geq 99,9\%$, trypsin-EDTA solution
229 10X, formaldehyde solution for molecular biology $\geq 36.0\%$, 4',6-diamidino-2-phenylindole dilactate
230 (DAPI) for nuclei staining, Mowiol[®] 4-88, rhodamine B isothiocyanate, Hanks' Balanced Salt solution
231 (HBSS) and L-glutamine were purchased from Sigma-Aldrich (Milan, Italy). Dulbecco's Modified
232 Eagle Medium (DMEM 1X) was purchased from Gibco[®] brl life technologies[™] inc., Grand Island (NY,
233 USA). Lyso-Tracker Green DND-26, Invitrogen[™] Molecular probes[®] were purchased from Thermo
234 Fisher Scientific (Monza, Italy). Triton[®] X-100, albumin from bovine serum, ammonium formate,
235 trypan blue solution were purchased from Biochemica Fluka, Sigma-Aldrich (Milan, Italy).
236 Nitrotetrazolium blue chloride (MTT, CellTiter 96[®] Non-Radioactive Cell Proliferation Assay) was
237 purchased from Promega (Milan, Italy). Acetonitrile for HPLC and ACS water suitable for
238 UPLC/UHPLC instruments were purchased from VWR (Milan, Italy).

239 *Bacterial strains: Staphylococcus aureus* NCTC 12973 was obtained from Public Health England
240 Southampton, UK. Stock cultures were stored at -80°C using Protect cryobead vials (Technical
241 Service Consultants Ltd, Heywood UK). Prior to experiments bacterial cultures were revived by
242 transferring cryobead/s onto fresh sterile Mueller-Hinton agar and incubated at 37°C for 24 h.
243 Dehydrated bacteriological media were obtained from Oxoid (Basingstoke, Hampshire, U.K.) and
244 reconstituted according to the manufacturer's instructions. Bacterial growth media were sterilized
245 at 121°C , 15 psi for 15 min prior to use.

246 *General cell culture:* The human keratinocyte cell line (HaCaT) was spontaneously immortalized from
247 primary keratinocytes^[35] and cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM;
248 EuroClone, Milan, Italy) supplemented with 10% (v/v) fetal bovine serum (FBS) and 4 mM glutamine
249 at 37°C in a humidified atmosphere with 5% CO_2 . Cells were grown to 70-80% semi-confluence,
250 according to each experimental setting and treated with free or loaded antibiotics at several time
251 points and concentrations. All experiments contained untreated cells (PBS) processed in parallel as
252 negative control.

253

254 *Synthesis of cholesterol-Br-butyric derivative (CH-Br):* CH-Br synthesis was carried out as previously
255 described [29]. Briefly, 500 mg of CH (1.3×10^{-3} mol) were solubilized in 5 mL of CH_2Cl_2 and added to
256 79 mg of DMAP (6.5×10^{-4} mol); the solution was stirred for 15 min at 25°C. Meanwhile, 648 mg of
257 4-bromobutyric acid (3.9×10^{-3} mol) and 744 mg of EDC·HCl (3.9×10^{-3} mol) were solubilized in 5 mL
258 of CH_2Cl_2 ; the two solutions were then mixed and kept at 25°C with magnetic stirring, overnight.
259 Reaction products were checked with silica gel TLC (cyclohexane : ethylacetate, 85:15) and then
260 washed once with 50 mM NaOH and HCl and three times with bi-distilled water. The solution was
261 firstly dried with anhydrous Na_2SO_4 and then with vacuum evaporation. The crude product (powder)
262 was finally purified with a silica column (eluent, cyclohexane : ethylacetate, 98.5:1.5); 310 mg of
263 pure CH-Br were obtained (yield 60%, % w/w).

264

265 *Synthesis of hyaluronan-cholesterol amphiphilic polymer (HA-CH):* HA-CH synthesis was carried out
266 as previously reported [29]. 200 mg of $\text{HA}^- \text{TBA}^+$ ($M_w = 2.2 \times 10^5$) were added to 10 mL of NMP and the
267 sample was kept at 25°C for 5 h with magnetic stirring; then, 34.3 mg of CH-Br, previously solubilized
268 in 2 mL of NMP, were added and the reaction was allowed to proceed for 48 h at 38°C with magnetic
269 stirring. Then, 2 mL of NaCl (saturated solution) were added to the polymer solution and the mixture
270 was left under stirring for 30 min to allow the exchange of the Na^+ with TBA^+ ions. The reaction
271 product was precipitated in acetone (4 times the reaction volume), left for 1 h at 4°C, isolated, re-
272 suspended in bi-distilled water and finally dialysed against water (cellulose membrane tubing, M_w
273 cut-off: $1.2\text{-}1.4 \times 10^4$, Sigma-Aldrich, Darmstadt, Germany) until constant conductivity was reached.
274 Sample was freeze-dried with a "Modulyo 4K" Edwards High Vacuum instrument, equipped with an
275 Edwards pump, operating at 0.2 atm and at -40°C, yielding 354 mg of white solid (71% mass
276 recovery). Theoretical degree of functionalisation (DF) was 15% (% mol/mol, corresponding to mol
277 of CH per mol of HA repeating unit).

278

279 *Preparation and characterization of GM or LVF-loaded HA-CH nanohydrogels (NH/GM or NH/LVF):*

280 2 mg of HA-CH were dispersed in 1 mL of Milli-Q water (2 mg mL⁻¹) overnight with magnetic stirring
281 at 25°C. Then 1 mL PBS (pH=7.4) and 0.1 mL of GM solution (10, 5 or 2.5 mg mL⁻¹), or 1 mL Milli-Q
282 water and 0.1 mL of LVF solution (10, 5 or 2.5 mg mL⁻¹) (corresponding to 1:1, 1:0.5 and 1:0.25 HA-
283 CH:GM or HA-CH:LVF wt. ratio) were added. Samples were then autoclaved for 20 min at 121°C,
284 leading to NH/GM or NH/LVF formation. NH/GM or NH/LVF suspensions were purified from the free
285 antibiotics through SEC. SEC was performed using Econo-Pac chromatography columns (Bio-Rad,
286 Segrate, Italy) packed with Bio-Gel® P-10 (polyacrylamide with an exclusion limit range of 1.5-20 x
287 10³, Bio-Rad, Segrate, Italy). 1 mL of NH/GM or NH/LVF mixtures was loaded into SEC and eluted
288 with 10 mL PBS or water, respectively, at 25°C and atmospheric pressure.

289

290 *Dynamic Light Scattering (DLS):* hydrodynamic diameter (Z-average size), size distribution,
291 polydispersity (PDI), and ζ-potential of NH/GM or NH/LVF were measured by Dynamic Light
292 Scattering (DLS) at 25 °C by using a Zetasizer Nano ZS instrument (Model ZEN3690, Malvern
293 Instruments) equipped with a solid state HeNe laser (λ = 633 nm) at a scattering angle of 173°. Size
294 measurement data were analyzed by using the general purpose algorithm. The electrophoretic
295 mobility of the samples was converted in ζ-potential by using the Smoluchowski equation.
296 Hydrodynamic diameter and PDI of freeze-thawed or freeze-dried NH/GM or NH/LVF were also
297 studied; empty NHs were prepared as controls. Each experiment was performed in triplicate (n=3).

298

299 *Quantification of entrapped GM or LVF into NHs:*

300 A) *UPLC coupled to mass spectrometry (LC-MS) analysis:* the amount of GM entrapped into NHs
301 was checked with a Waters Acquity H-Class UPLC liquid chromatography-mass spectrometer
302 (Waters, Milford, MA, USA), equipped with a quaternary solvent manager (QSM), a sample

303 manager with flow through needle system (FTN) and a single-quadrupole mass detector with
304 electrospray ionization source (ACQUITY QDa). Free GM solutions were diluted with water,
305 filtered with 0.22 μm regenerated cellulose membrane filters (Sartorius Italy s.r.l., Monza,
306 Italy) and injected into a Waters BEH Shield C18 column (50 mm \times 2.1 mm i.d., 1.7 μm particle
307 size). Samples were eluted using an isocratic 10 mM HCOONH_4 mobile phase (pH = 3) at a
308 flow rate of 0.5 mL min^{-1} and column temperature of 25°C. Mass spectrometry detection
309 was performed in positive electrospray ionization mode (ESI), using nitrogen as nebulizer
310 gas. Three m/z values (478, 464 and 450) corresponding to the three $[\text{M}+\text{H}]^+$ GM chemical
311 species were monitored in single ion recognition mode (SIR). Capillary and cone voltage were
312 set to 0.8 kV and 15 V; ion source and probe temperatures were adjusted to 120°C and
313 600°C. A GM calibration curve was calculated at the concentration range of 7.5-125 $\mu\text{g mL}^{-1}$
314 ($R^2 = 0.98$, $n = 3$).

315 B) *UV-Vis spectrometry analysis*: the amount of LVF entrapped into NHs was assessed by using
316 a Perkin-Elmer double beam "Lambda 3A" model. Analyses were performed at 25°C, using 1
317 mm quartz cuvettes (Hellma Analytics, Milan, Italy). LVF water solutions were detected at
318 286 nm. A LVF calibration curve was built at the concentration range of 16-200 $\mu\text{g mL}^{-1}$ ($R^2 =$
319 0.999 , $n=5$). The amount of entrapped GM or LVF was calculated by subtracting the amount
320 of unloaded GM or LVF from the starting total amount of drugs. Each experiment was
321 performed in triplicate ($n=3$).

322 Drug loading (DL%) and loading efficiency (LE%) of both GM and LVF were calculated using the
323 following equation (1) and (2):

324

$$325 \quad DL\% = \frac{\text{weigh of loaded drug}}{\text{weig of total drug}} \times 100 \quad (1)$$

326

327
$$LE\% = \frac{\text{weight of loaded drug}}{\text{weight of polymer}} \times 100 \quad (2)$$

328

329

330 *In vitro release studies of GM or LVF from NHs:* 1 mL of purified NH/GM or NH/LVF (1:1 starting wt.
331 ratio) was put into a membrane tube (Mw cut-off $1.2-1.4 \times 10^4$) and dialysis was performed against
332 15 mL bi-distilled water for 24 h, at 37°C; at specific time points 3 mL solution were taken off and
333 replaced with 3 mL of bi-distilled water. Samples were then injected into UPLC-MS or analysed with
334 UV-Vis spectrometer for quantifying the amount of released GM or LVF from NHs, respectively. Each
335 experiment was performed in triplicate (n=3).

336

337 *Cell viability assays:* HaCaT viability was tested in parallel with 3-(4,5-dimethylthiazol-2-yl)-2,5-
338 diphenyltetrazolium bromide metabolic assay (MTT assay) and trypan blue exclusion method, by
339 counting both dead and living cells.

340 A) *MTT test:* 100 μ L HaCaT (5,000 cells/well in complete DMEM) were seeded in a 96-well plate
341 (Falcon™ Polystyrene Microplates, Thermo Fisher Scientific, Monza, Italy) and incubated for
342 24 h. Cell monolayers were then added to 25 μ L of samples (free NHs, GM or LVF, NH/GM
343 or NH/LVF in PBS pH=7.4) at specific final NHs concentrations (ranging from 18-500 and 37-
344 1000 μ g mL⁻¹ for NH/GM and NH/LVF respectively, df: 1:3) and incubated for 24 and 48 h. As
345 negative control, cells received 25 μ L PBS. Then, medium was removed, cells were gently
346 washed with PBS and 100 μ L complete DMEM were added. 20 μ L MTT solution (Promega,
347 Italy, Milan) were added and HaCaT were incubated for 2 h. Supernatants were gently
348 removed and formazan crystals were solubilised with 100 μ L DMSO. Absorbance was
349 checked at 570 nm with a reference at 690 nm, using an Appliskan microplate reader
350 (Thermo Scientific, Vantaa, Finland). Each experiment was performed on sixteen wells (n=3).
351 Results were processed using SkanIt 2.3 Software.

352 B) *Trypan Blue exclusion method*: 1.5 mL HaCaT (167,000 cells/well in completed DMEM) were
353 seeded in a 6-well plate and incubated for 24 h. 0.375 mL of free NHs or NH/GM or NH/LVF
354 in PBS at the final NHs concentration of 500 or 1000 $\mu\text{g mL}^{-1}$ (NH/GM or NH/LVF respectively)
355 were then added to the cells, which were incubated for 24 and 48 h. As negative control,
356 cells received 0.375 mL PBS. Subsequently, medium (1.875 mL) was taken off and stored into
357 eppendorf tubes (Primo[®] boil-proof microcentrifuge tubes, EuroClone spA, Milan, Italy);
358 then, wells were gently washed with 1 mL PBS and 0.6 mL 0.5% trypsin were added. Cells
359 were allowed to detach for 10 minutes at 37°C and then added to the previously removed
360 1.875 mL DMEM. 80 μL cell suspensions were mixed with 80 μL 0.4% trypan blue solution
361 and cells were counted with a Primovert microscope (Carl Zeiss Microimaging GmbH,
362 Gottingen, Germany) in a Neubauer chamber. Each experiment was performed in triplicate
363 (n=3).

364

365 *Determination of MICs and MBCs:*

366 The MIC and MBC were determined for axenic planktonic populations of *S. aureus* using the
367 microdilution method ^[36]. Briefly, 100 μL double strength Mueller-Hinton broth was dispensed in
368 wells of the first column of a flat bottom 96-well microtiter plate. Stationary phase cultures of *S.*
369 *aureus* grown in Mueller-Hinton broth, were adjusted in fresh sterile Mueller-Hinton broth to give
370 a cell density of c. 2×10^6 CFU mL^{-1} . Within 30 minutes of preparation inocula were dispensed (100
371 μL) into the remaining wells the 96-well plate. Stock solutions of free GM, LVF, NHs or NH/GM or
372 NH/LVF were prepared in sterile PBS and dispensed (100 μL) into the first column of the microtiter
373 plate. Doubling dilutions of samples were then undertaken across the plate (100 μL). Plates were
374 incubated at 37°C for 24 h. The MIC endpoint was determined as the lowest concentration of
375 antimicrobial that prevented visible growth. To determine the MBC, aliquots (10 μL) from wells
376 exhibiting no turbidity were spot plated onto sterile Mueller-Hinton agar. After incubation at 37°C,

377 the MBC endpoint was determined as the lowest concentration that resulted in no visual growth of
378 colonies after 24 h of incubation. Each experiment was performed in triplicate (n=3).

379

380 *Cell infection and assessment of intracellular activity of NH/GM or NH/LVF:* To assess the efficacy of
381 free or loaded GM or LVF on intracellular bacteria, HaCaT cell monolayers were grown to 70-80%
382 semi-confluence by seeding 1.5×10^5 cells in 1 mL of complete DMEM (2 mM glutamine) in 24-well
383 plates and incubating for 18 h. Mid-log phase cultures of *S. aureus* were washed twice with sterile
384 PBS and culture densities adjusted in sterile, FCS free DMEM to give a cell density of c. 100 CFU/cell
385 (100 MOI). Growth medium was removed and HaCaT cells were washed with sterile PBS and FCS
386 free medium was added to each well. Aliquots of adjusted bacterial cultures were added to cells
387 and plates were incubated for a further 3 h. In order to remove any extracellular bacteria, cell
388 culture media were then removed from wells and 1 mL fresh serum free media containing X2 MIC
389 gentamicin added. Plates were further incubated for 1 h. Medium was removed and HaCaT cells
390 were washed twice with sterile PBS. Free GM or LVF and their nano-formulations were reconstituted
391 in sterile PBS and diluted to the desired concentration in fresh serum free DMEM in order to have
392 3X MIC value (final concentration of drugs in the well). 1 mL aliquots of treatments were then added
393 to cells and plates were incubated for either 2 or 5 h. After incubation, cells were washed twice with
394 PBS and lysed with 0.025% Triton X for 10 min. To enumerate viable bacteria, lysates were harvested
395 and serially diluted in sterile PBS and inoculated onto Mueller-Hinton agar. Agar plates were
396 incubated for > 16 h and viable counts performed. All experiments contained non-infected cell
397 monolayers processed in parallel as a sterile control and infected cell monolayers processed with
398 serum free media in the absence of any test treatment as a positive control. Each experiment was
399 performed in triplicate (n=3).

400

401 *Synthesis of fluorescent NHs (rhod-NHs):* 1.5 mg mL⁻¹ HA-CH polymer in bi-distilled water was left
402 with magnetic stirring overnight at 25°C. The sample was placed in an autoclave (121°C for 20 min)
403 where NHs were allowed to form. Rhodamine B-isothiocyanate (rhod) was previously solubilized in
404 DMSO at a concentration of 9 mg mL⁻¹ and then added to NHs suspension (8 µL for 1 mg of polymer,
405 corresponding to a DF of 6.3%; % mol/mol). The reaction mixture was left for 5 h at 25°C in the dark,
406 following which it was dialysed against water until constant conductivity was reached and then
407 freeze-dried. The final DF% was assessed through UV-Vis analysis: sample was solubilized in DMSO
408 and checked at 550 nm at 25°C. A rhod calibration curve was built at the concentration range of 8.5-
409 125 µg mL⁻¹ in DMSO. DF corresponded to 1.3% (mol of rhod per mol of HA-CH repeating unit).

410

411 *Flow cytometry analysis:* Cell binding/uptake kinetics of fluorescent NHs was studied with a BD
412 Accuri C6, BD 254 Biosciences (Erembodegem, Belgium) flow cytometer equipped with a 488 nm
413 excitation laser and a 585/40 nm band-pass filter (FL2 channel). For each sample 50,000 events were
414 collected. 1 mL HaCaT cell suspension (67,000 cells/well in complete DMEM) was seeded in 12-well
415 plates and incubated for 48 h. Cells were washed three times with PBS, 1.5 mL of complete DMEM
416 added and treated with 0.25 mL of 0.5 mg mL⁻¹ rhod-NHs in PBS (corresponding to a final
417 concentration of 0.10 mg mL⁻¹) at specific time points, over 24 h. Medium was removed, cells were
418 washed three times with PBS, allowed to detach with 0.25 mL trypsin and finally added to 0.75 mL
419 complete DMEM. Cell suspensions were centrifuged in eppendorf tubes for 8 min at 1,200 rpm at
420 4°C. Supernatants were removed and pellets were washed with 1 mL HBSS solution and centrifuged
421 again. Pellets were re-suspended in 0.5 mL HBSS and red fluorescence (rhod) was detected with a
422 flow cytometer. HaCaT cells were also treated with 0.25 mL of rhod-NHs (final concentrations 10,
423 50, 100, 500 µg mL⁻¹) at a fixed time point (4 h) by following the same procedure and then analysed
424 with a flow cytometer. As a negative control, cells received 0.25 mL PBS. Results are expressed as
425 median fluorescence intensity. (n=3). A dot plot was built by plotting Forward scatter (FSC-H) versus

426 Side scatter (SSC-H) and the gate was constructed excluding cell debris. Each experiment was
427 performed in triplicate (n=3).

428

429 *Cell imaging:*

430 *ApoTome microscope:* Immunofluorescence signal of fluorescent NHs was analysed by recording
431 stained images using an Axio Observer Z1 inverted microscope, equipped with an ApoTome.2
432 System (Carl Zeiss Inc., Ober Kochen, Germany). The ApoTome system provides an optical section
433 of fluorescent samples, calculated from three images with different grid positions without time lag.
434 Digital images were acquired with the AxioCam MRm high resolution digital camera (Zeiss) and
435 processed with the AxioVision 4.8.2 software (Zeiss). ApoTome optical sectioning images of
436 fluorescent NHs were recorded under 40x/0.75 objective (Zeiss). Pearson's correlation coefficient
437 ^[32] was used to quantify the degree of colocalization between fluorescent NHs and Lyso-Tracker
438 Green DND-26 staining in a series of 0.5 nm sequential sections, under an immersion oil 63x/1.25
439 objective (Zeiss). Pearson's correlation coefficient was calculated using the AxioVision 4.8.2
440 software (Zeiss), analyzing a minimum of 40 cells randomly taken from each slide from three
441 independent experiments. Images were obtained from the 2D reconstruction of selected serial
442 optical sections.

443

444 *Cellular uptake micro-graphs:* 1.5 mL HaCaT cells (167,000 cells/well in complete DMEM) were
445 seeded on microscope slides (Prestige micro cover glass 22 X 22 mm, Syntesys Disposable Labware,
446 Padova, Italy) in a 6-well plate and allowed to adhere for 48 h. Then, cells were washed three times
447 with PBS, 1.5 mL of complete DMEM added and incubated with 0.375 mL of 0.5 mg mL⁻¹ rhod-NHs
448 in PBS (corresponding to a final concentration of 0.1 mg mL⁻¹) or free rhod previously solubilised in
449 DMSO (final concentration of 7.2 µg mL⁻¹). After 4 h, medium was removed and cells were washed
450 three times with PBS. Cells were fixed with 2% (v/v) formaldehyde (2 mL, 10 min), washed three

451 times with PBS and incubated with Triton X-100 (0.1%, v/v, 2 mL, 5 min). Cells were washed three
452 times with PBS and nuclei were stained with DAPI reagent ($1 \mu\text{g mL}^{-1}$, 1 mL for 1 min). Finally, cells
453 were washed again and 8 μL Mowiol were added; slides were allowed to dry and fix overnight at
454 25°C on StarFrost[®] microscope slides (Braunschweig, Germany). Samples were then analysed with
455 the ApoTome microscope.

456

457 *Lysosomal co-localization in living cells:* 1.5 mL HaCaT (167,000 cells/well in complete DMEM) were
458 seeded on microscope slides in a 6-well plate and allowed to adhere for 48 h. Cells were washed
459 three times with PBS, 1.5 mL of complete DMEM added and incubated with 0.375 mL of 0.5 mg mL^{-1}
460 1 rhod-NHs (corresponding to a final concentration of 0.1 mg mL^{-1}). After several time points (from
461 0.5 to 24 h) slides were washed three times with PBS, 1.5 mL of complete DMEM added and finally
462 incubated with 1 mL of Lyso-Tracker Green DND-26 staining (final concentration of 200 nM) for 3
463 min at 37°C . Images were immediately recorded on living cells with the ApoTome microscope.
464 Results were obtained from three independent experiments, each derived from eight images.

465

466 *Statistical analysis:*

467 *Cell infection and assessment of intracellular activity of NH/GM or NH/LVF.*

468 Viable cell counts were calculated using three biological replicate count data (each derived from
469 three technical replicate data). All data were Log₁₀ transformed and are expressed as the mean
470 value \pm standard deviation. Statistical significance was determined using biological replicate data
471 ($n=3$) with Mann-Whitney test by using SPSS 20 Software. P values < 0.05 were considered
472 significant. Asterisk denote statistically significant differences ($*P<0.05$).

473 *Cell viability and cell count assays*

474 Viable HaCaT (MTT and trypan blue) were calculated using three biological replicates (each derived
475 from sixteen wells). Cell viability and cell growth were normalized to the negative control (untreated

476 cells that received PBS), whilst the cell death was normalized to the total amount of cells. All data
477 are expressed as the mean value \pm standard deviation. Statistical significance was determined using
478 sixteen wells (n=3) with One-way ANOVA analysis in Prism (GraphPad 5.0 Software, Inc., La Jolla, CA,
479 USA). Differences between groups were determined by a Turkey's multiple comparison test.
480 Asterisks denote statistically significant differences (*P<0.05; **P<0.01; ***P<0.005).

481 *Co-localization experiments*

482 Pearson's correlation coefficient was used to quantify the degree of co-localization between
483 fluorescent NHs and Lyso-Tracker Green DND-26 staining, which was calculated using the AxioVision
484 4.8.2 software (Zeiss) and expressed as the mean value \pm standard deviation. Results were obtained
485 from three independent experiments, each derived from eight images. Statistical significance was
486 determined using One-way ANOVA analysis.

487

488 *Graphs and Figures:* All graphs and figures were processed with OriginPro 2016 and CorelDraw
489 Graphics Suite X8 Softwares.

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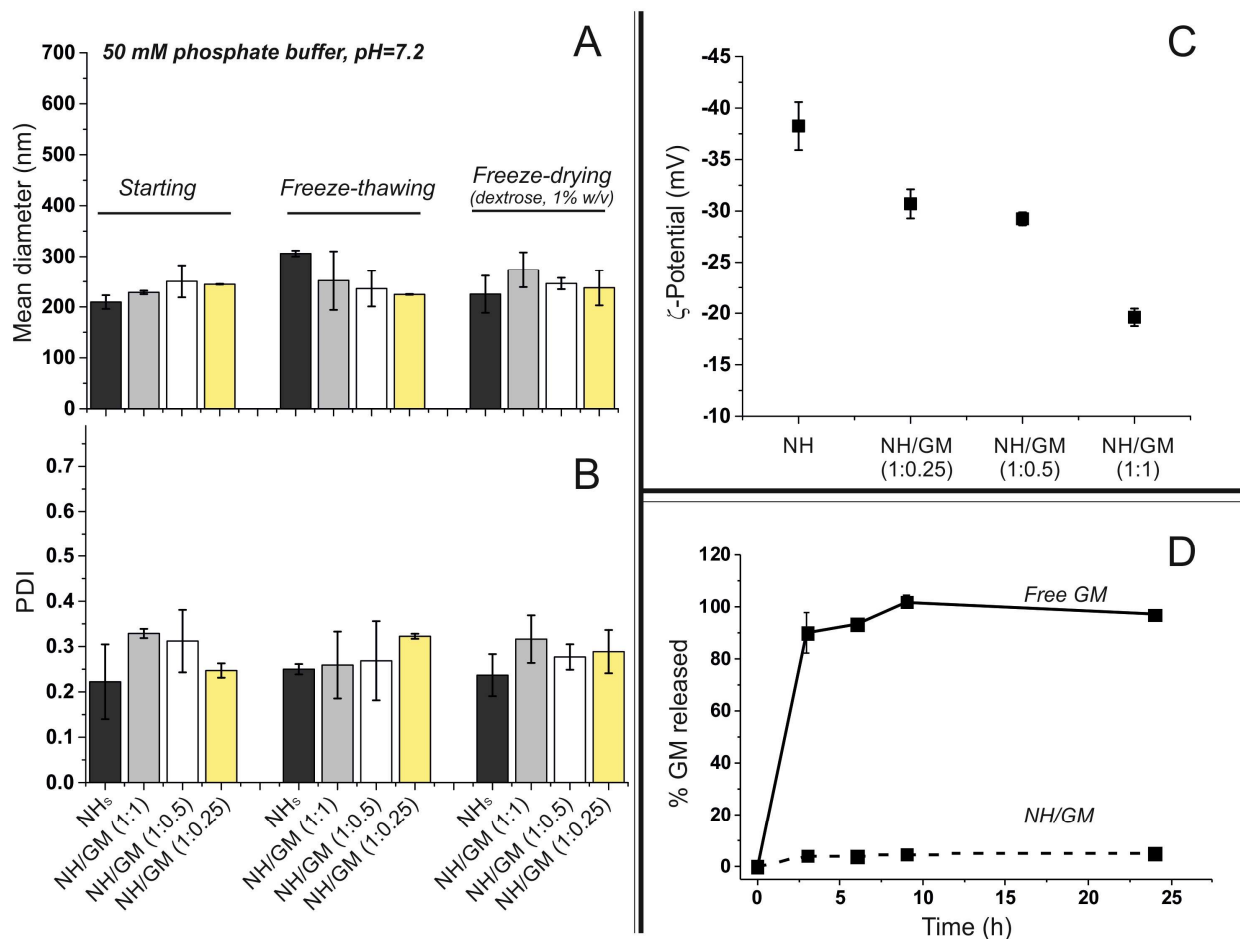


Figure 1. Mean diameter (A) and PDI (B) of starting, freeze-thawed and freeze-dried NH/GM. HA-CH/GM mixtures were prepared at several wt. ratios (ranging from 1:0.25 to 1:1 of HA-CH and GM, respectively) in 50 mM PBS (pH=7.4)

and then autoclaved at 121°C for 20 min. Unloaded GM was discarded by purification through a SEC column. (C) ζ -pot of NH/GM samples. (D) Release of GM from NHs over 24 h at 37°C in bi-distilled water. Released GM was analysed though UPLC coupled with mass-spectrometer. All data are expressed as the mean value \pm standard deviation. Results were obtained in triplicate (n=3).

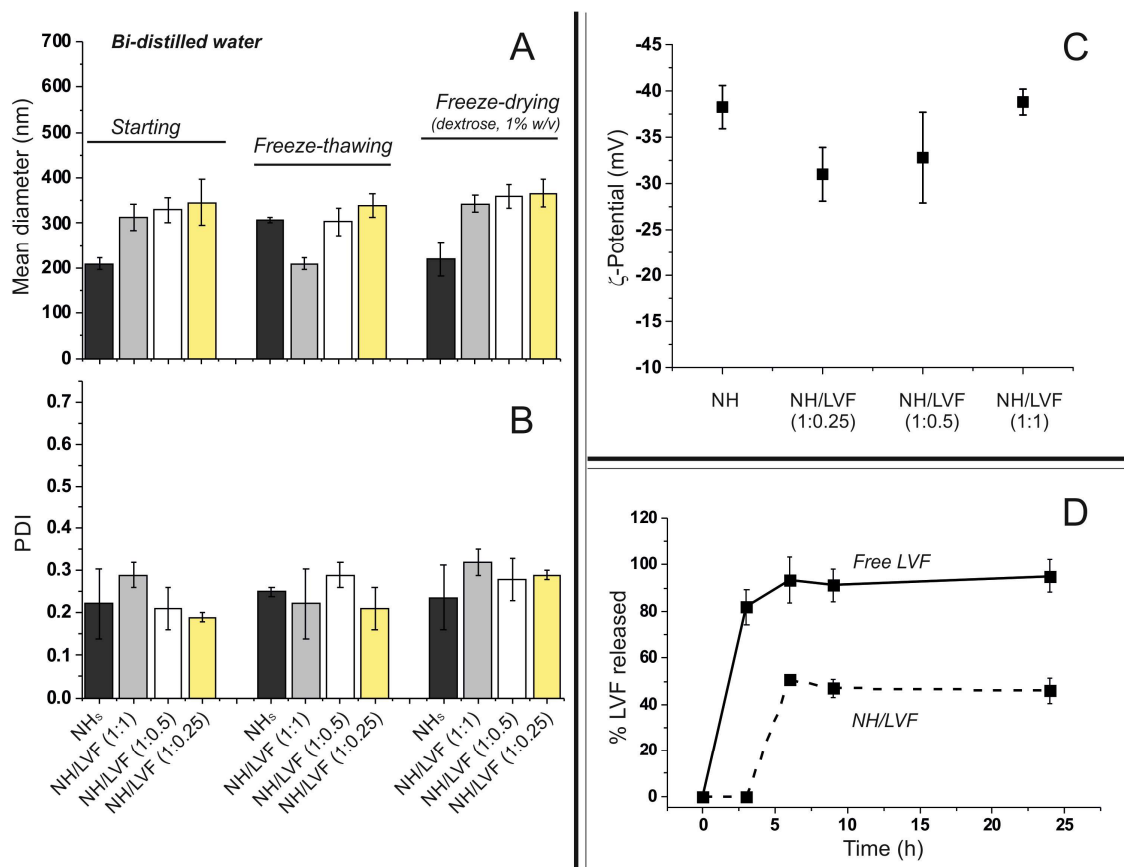


Figure 2. Mean diameter (A) and PDI (B) of starting, freeze-thawed and freeze-dried NH/LVF. HA-CH/LVF mixtures were prepared at several wt. ratios (ranging from 1:0.25 to 1:1 of HA-CH and LVF, respectively) in bi-distilled water and then autoclaved at 121°C for 20 min. Unloaded LVF was discarded by purification through a SEC column. (C) ζ -pot of NH/LVF samples and (D) release of LVF from NHs over 24 h at 37°C in bi-distilled water. LVF was detected with UV-Vis spectrometer. All data are expressed as the mean value \pm standard deviation. Results were obtained in triplicate (n=3).

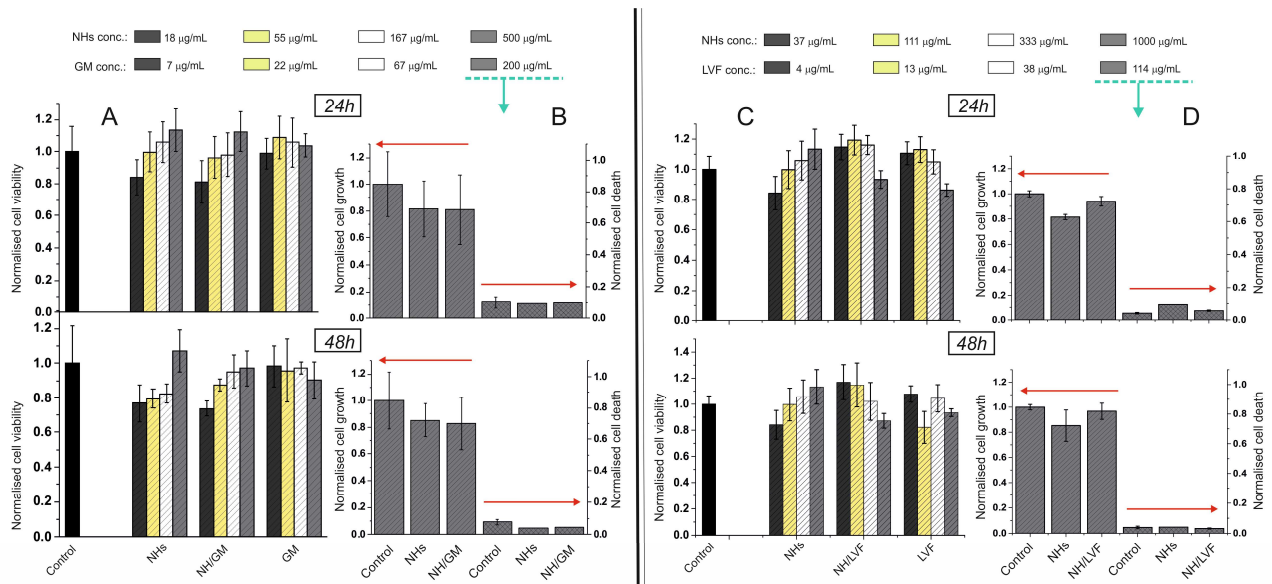


Figure 3. Viability of HaCaT: MTT (A and C) and trypan blue (B and D) assays were performed on cells cultured in completed DMEM, incubated for 24 and 48 h with NH/GM (A and B) and NH/LVF (C and D). Only samples with the starting 1:1 wt. ratio (HA-CH:drug) were tested. Results were obtained from three independent experiments (each derived from sixteen wells). All data are expressed as the mean value \pm standard deviation. MTT and cell growth results were normalized to the control (untreated cells that received PBS), whilst the cell death was normalized to the total amount of cells. Statistical significance was determined with One-way ANOVA analysis. Differences between groups were determined by a Turkey's multiple comparison test. No significant differences were detected.

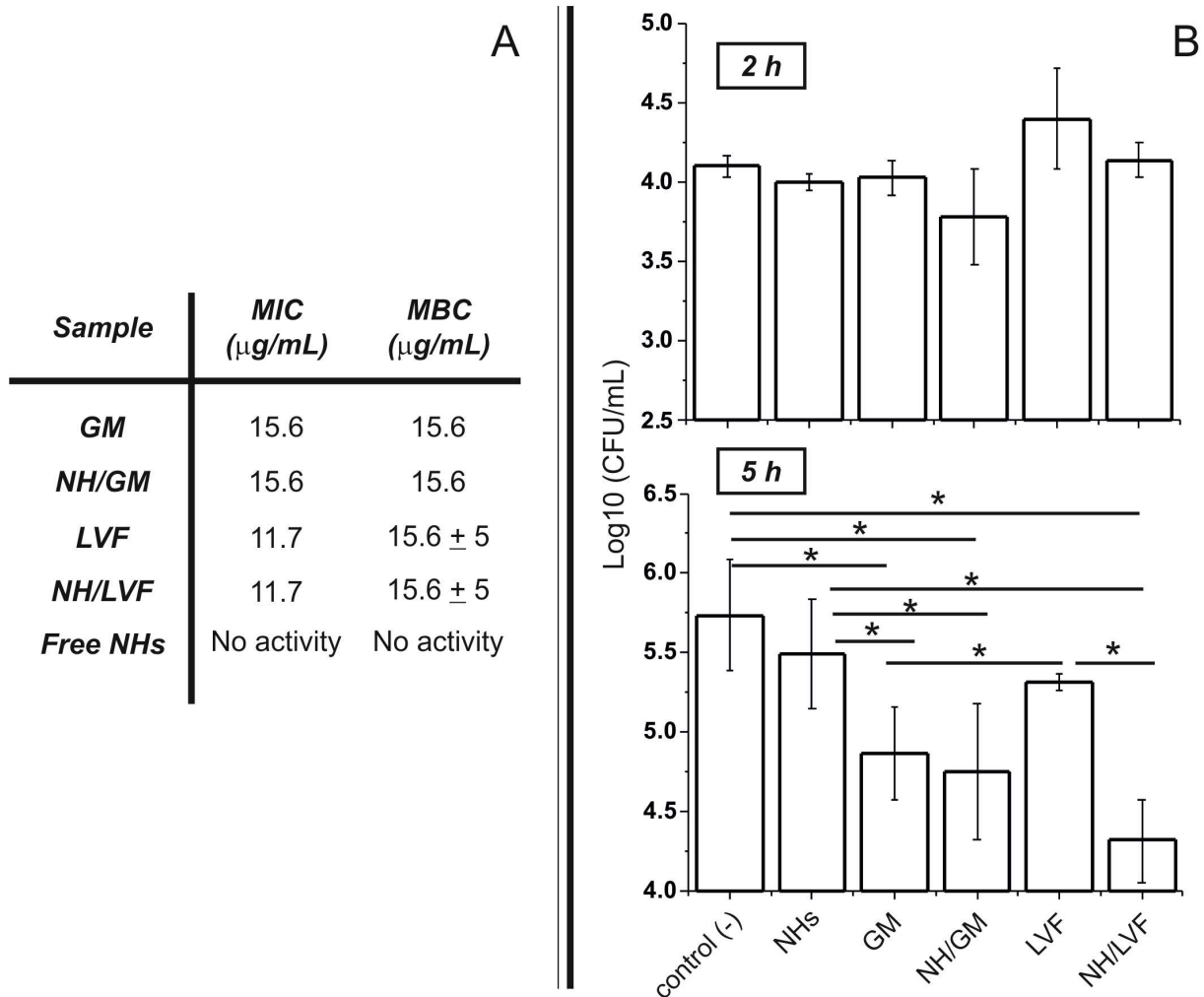


Figure 4. (A) MIC and MBC of NH/GM, NH/LVF and their controls against planktonic *S. aureus*. Plates were incubated for 24 h. The MIC endpoint was determined as the lowest concentration of antimicrobial at which there was no visible growth; the MBC endpoint was determined as the lowest concentration that resulted in no visual growth of colonies after 24 h of incubation. (B) Intracellular activity of NH/GM, NH/LVF and their controls against *S. aureus*-infected HaCaT. Viable cell counts were calculated using three biological replicate count data (each derived from three technical replicate data). All data were Log₁₀ transformed and are expressed as the mean value \pm standard deviation. Statistical significance was determined using biological replicate data (n=3) with Mann-Whitney test by using SPSS 20 Software. P values < 0.05 were considered significant. Asterisk denote statistically significant differences (*P<0.05).

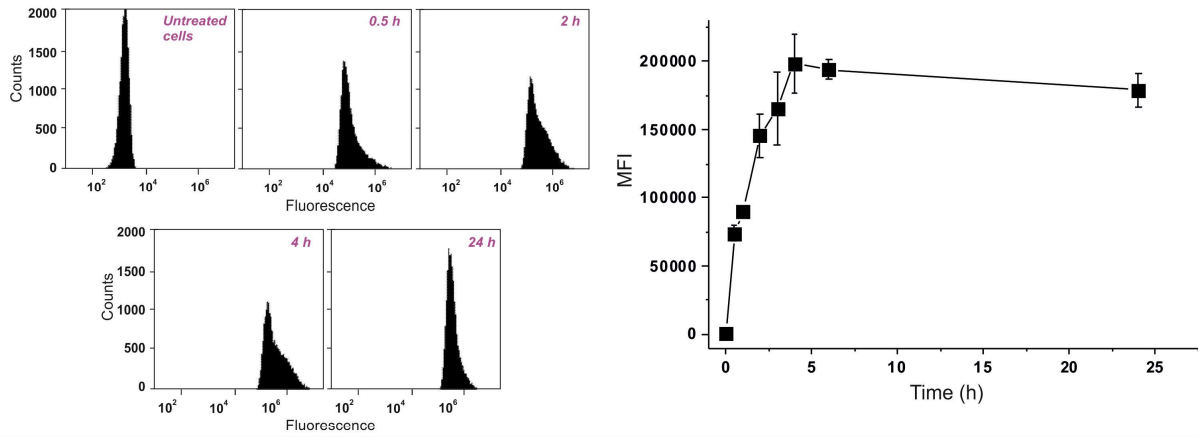
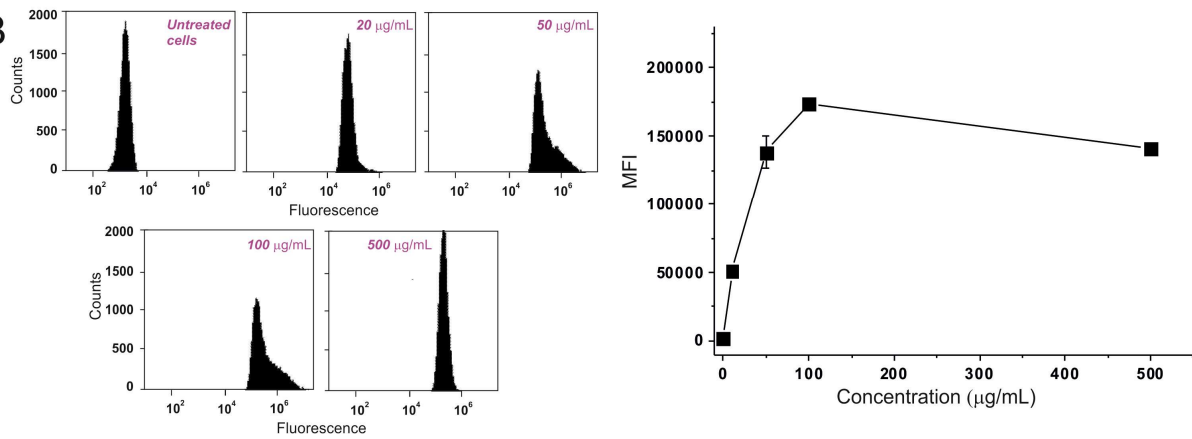
A**B**

Figure 5. Flow cytometry analysis of HaCaT cells incubated with rhod-NHs. Cells were cultured in complete DMEM for 48 h and then incubated from 0.5 to 24 h with 100 $\mu\text{g mL}^{-1}$ rhod-NHs (A) or with 20-500 $\mu\text{g mL}^{-1}$ of rhod-NHs for 4 h (B). Results were obtained in triplicate (n=3). All data are expressed as the mean value \pm standard deviation.

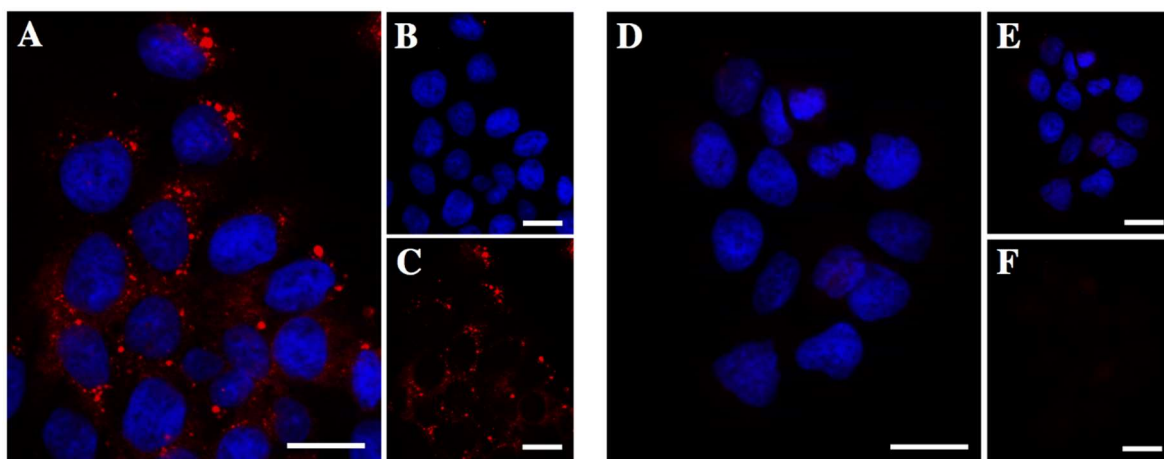


Figure 6. ApoTome micro-graphs (scale bars: 20 μm) of HaCaT cells incubated with 100 $\mu\text{g mL}^{-1}$ rhod-NHs (A (merge), B (DAPI), C (rhod-NHs) or free rhod (D (merge), E (DAPI), F (rhod))). Cells were cultured in complete DMEM for 48 h and then incubated for 4 h with fluorescent nanoparticles.

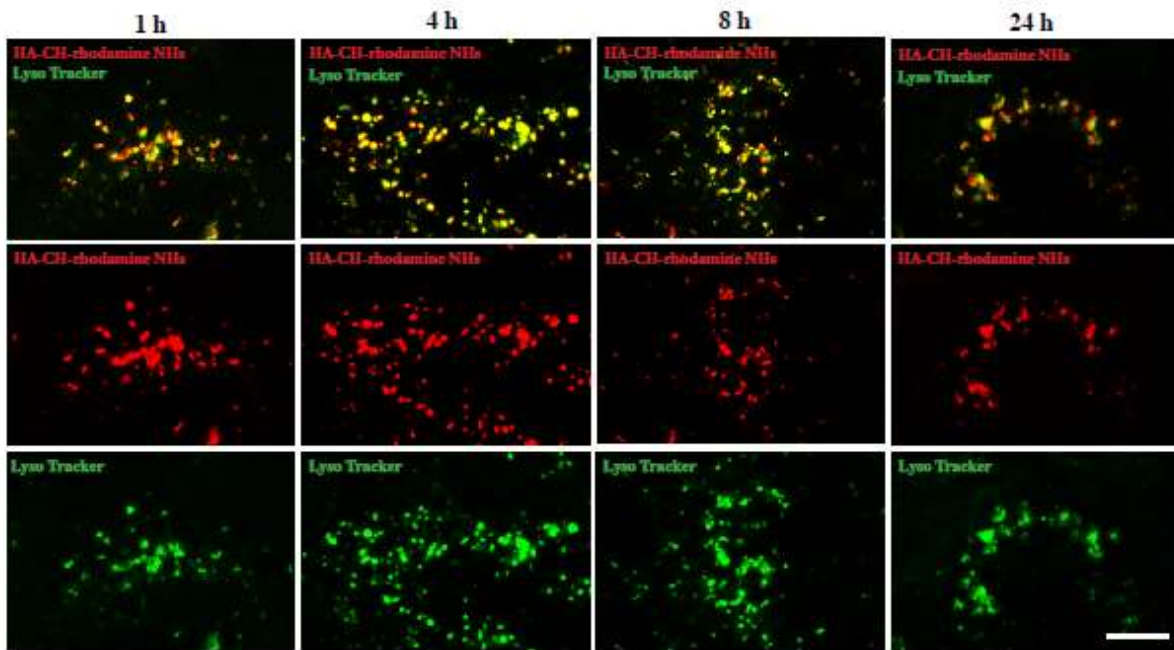
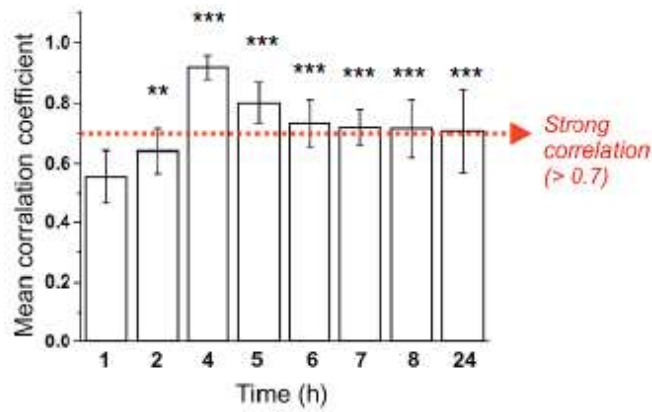


Figure 7. ApoTome micro-graphs (scale bar: 10 μm): co-localization with lysosomes. HaCaT cells were cultured in complete DMEM for 48 h and then incubated from 1 to 24 h with 100 $\mu\text{g mL}^{-1}$ rhod-NHs. After specific time points slides were washed with PBS, added again to 1.5 mL of complete DMEM and finally treated with 1 mL of Lyso-Tracker Green' (final conc. of 200 nM) for 3 min at 37°C. Images were immediately recorded on living cells. Pearson's correlation coefficient was calculated using the AxioVision 4.8.2 software (Zeiss) and expressed as the mean value \pm standard deviation. Results were obtained from three independent experiments, each derived from eight images. Statistical significance was determined with One-way ANOVA analysis. Differences between groups were determined by a Turkey's multiple comparison test. Asterisks denote statistically significant differences with the incubation time of 1 h (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$).

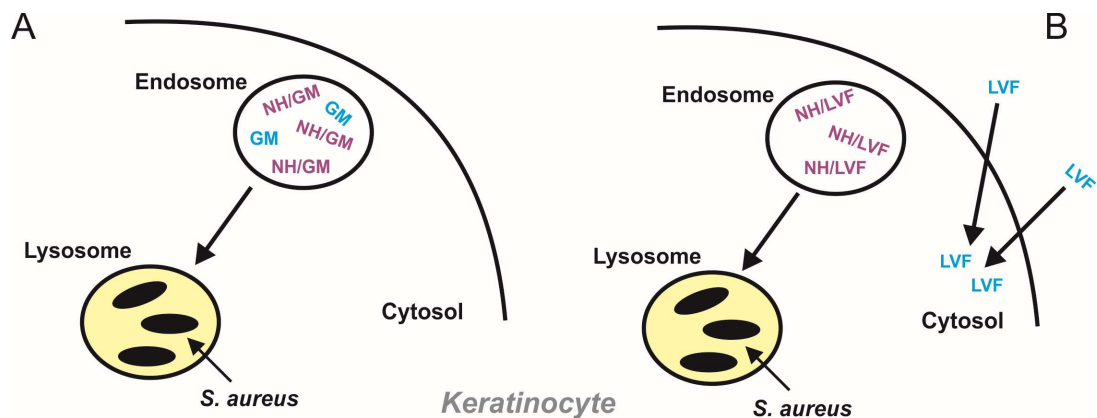


Figure 8. Scheme of the intracellular fate of free GM (A), LVF (B) and their nano-formulations in *S. aureus*-infected keratinocytes.

Table 1. Drug loading (DL, %) and loading efficiency (LE, %) of NH/GM and NH/LVF prepared at several HA-CH:GM or HA-CH:LVF wt. ratios (ranging from 1:1 to 1:0.25). Nano-systems highlighted (bold) were chosen for the biological and microbiological analyses. Experiments were performed in triplicate (n=3) and results are expressed as the mean value \pm standard deviation.

Sample	DL%	LE%
NH/GM (1:1)	40.0 \pm 1.0	40.0 \pm 1.0
NH/GM (1:0.5)	35.1 \pm 2.8	17.5 \pm 1.4
NH/GM (1:0.25)	30.7 \pm 2.0	7.7 \pm 0.5
NH/LVF (1:1)	11.4 \pm 3.1	11.4 \pm 3.1
NH/GM (1:0.5)	9.5 \pm 2.0	4.74 \pm 1.0
NH/GM (1:0.25)	7.9 \pm 0.8	2.0 \pm 0.2