Manconi, M.; Manca, M.; Caddeo, C.; Cencetti, C.; Di Meo, C.; Zoratto, N.; Nácher Alonso, A.... (2018). Preparation of gellan-cholesterol nanohydrogels embedding baicalin and evaluation of their wound healing activity. European Journal of Pharmaceutics and Biopharmaceutics. 127:244-249. https://doi.org/10.1016/j.ejpb.2018.02.015

Preparation of gellan-cholesterol nanohydrogels embedding baicalin and evaluation of their wound healing activity

Maria Manconi [1], Maria Letizia Manca [1,*], Carla Caddeo [1], Claudia Cencetti [2], Chiara di Meo [2], Nicole Zoratto[2], Amparo Nacher [3,4], Anna Maria Fadda [1], Pietro Matricardi [2]

[1] Dept. of Scienze della Vita e dell'Ambiente, University of Cagliari, Cagliari, Italy

[2] Dept. of Drug Chemistry and Technologies, Sapienza, University of Roma, Roma, Italy

[3] Dept. of Pharmacy, Pharmaceutical Technology and Parasitology, University of Valencia, Burjassot, Spain

[4] Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València - Universitat de València, Burjassot, Spain

*Corresponding author:

Maria Letizia Manca

Tel.: +390706758582

E-mail address: mlmanca@unica.it

ABSTRACT

In the present work, the preparation, characterization and therapeutic potential of baicalin-loaded nanohydrogels are reported. The nanohydrogels were prepared by sonicating (S nanohydrogel) or autoclaving (A nanohydrogel) a dispersion of cholesterol-derivatized gellan in phosphate buffer. The nanohydrogel obtained by autoclave treatment showed the most promising results: smaller particles (~362 nm *vs.* ~530 nm), higher homogeneity (polydispersity index = ~0.24 *vs.* ~0.47), and lower viscosity than those obtained by sonication. *In vitro* studies demonstrated the ability of the nanohydrogels to favour the deposition of baicalin in the epidermis. A high biocompatibility was found for baicalin-loaded nanohydrogels, along with a great ability to counteract the toxic effect induced by hydrogen peroxide in cells, as the nanohydrogels re-established the normal conditions (~100% viability). Further, the potential of baicalin-loaded nanohydrogels in skin wound healing was demonstrated *in vivo* in mice by complete skin restoration and inhibition of specific inflammatory markers (i.e., myeloperoxidase, tumor necrosis factor-α, and oedema).

Keywords: baicalin; gellan, nanohydrogel, antioxidant activity, fibroblasts, wound healing.

1. INTRODUCTION

Gellan is a linear anionic polysaccharide showing a repeating unit based on a tetrasaccharide of β-1,3-d-glucose, β-1,4-d-glucuronic acid, and α-1,4-l-rhamnose [1]. It is water soluble and forms transparent hydrogels, even at low concentrations [2], by interaction with mono and divalent cations. The mechanism of gelation involves formation of double helical junction zones by the polymer strands, followed by aggregation of the hydrophobic double helical segments to form a three-dimensional network by complexation with cations and hydrogen bonding with water [3].

Gellan, even if useful in the native state, can benefit from a chemical conjugation with lipophilic molecules, e.g. cholesterol. In this case, its solubility in aqueous environment is altered, leading to an insoluble material. This new property can be exploited in order to shape a new drug delivery vehicle with soft hydrogel characteristics [4]. More precisely, the chemical derivatization of the carboxyl groups of gellan with a hydrophobic moiety is responsible for a self-assembling process resulting in the formation of nanosized particles capable of incorporating and delivering lipophilic molecules. Such nanohydrogel system was previously developed and tested for systemic administration of different drugs [4–6].

Baicalin (7-glucuronic acid 5, 6-dihydroxyflavone) is a flavone isolated from the roots of *Scutellaria baicalensis*, a medicinal plant widely used in East Asian countries for the treatment of various inflammatory diseases. The anti-inflammatory activity of baicalin has been attributed to its antioxidant properties and ability to inhibit nitric oxide and tumor necrosis factor-α (TNF-α) levels [7]. Recent studies reported the therapeutic properties of baicalin and its low bioavailability, especially when applied topically [8,9]. Hence, the incorporation of the polyphenol in nanocarrier systems can represent an effective approach to improve its bioavailability and modulate its release over time [10,11].

The use of nanohydrogels as topical delivery systems for baicalin was recently proposed [12].

In this work, the derivatization of gellan with cholesterol is presented, along with the preparation of nanohydrogels by using two techniques: sonication and autoclaving. The obtained nanohydrogels were characterized by size, zeta potential, rheological behaviour, and capability of delivering baicalin to the skin. Moreover, the biocompatibility and antioxidant activity of the baicalin loaded nanohydrogels were assessed *in vitro* using fibroblasts, and their potential in protecting the skin from chemically-induced injury was investigated *in vivo* in an animal model of chronic wound.

2. EXPERIMENTAL SECTION

2.1. Materials

Gellan tetrabutylammonium salt was kindly provided by Giusto Faravelli (Milan, Italy). Baicalin, cholesterol, 4-bromobutyric acid, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl), 12-O-tetradecanoylphorbol 13-acetate (TPA) and other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Phosphate buffer solution (PBS, pH 7) was purchased from Carlo Erba (Cornaredo, Milan, Italy). Cell medium, fetal bovine serum, penicillin/streptomycin, and fungizone were purchased from Life Technologies Europe (Monza, Italy). Betnovate® cream (betamethasone 0.1%; GlaxoSmithKline, Verona, Italy) was purchased in a local drugstore.

2.2. Gellan-cholesterol synthesis and nanohydrogel preparation

The synthesis of gellan-cholesterol derivative was carried out according to a previously developed procedure [5]. Briefly, cholesterol (500 mg, 1.3 mmol) and 4-(dimethylamino)pyridine (79 mg, 0.65 mmol) were solubilized in dichloromethane (7 ml). EDC-HCl (744 mg, 3.9 mmol) and 4-bromobutyric acid (648 mg, 3.9 mmol) were solubilized in dichloromethane (7 ml). The two solutions were mixed, kept at room temperature for 6 h, washed with NaOH (0.05 M), HCl (0.05 M) and distilled water (three times), and dried over anhydrous Na_2SO_4. The solvent was removed by rotary evaporation, and the crude was purified on silica column with ethylacetate:cyclohexane (15:85 v/v) leading to the Br-butyric-cholesterol derivative. Gellan tetrabutylammonium salt form

(1 g) was dissolved in *N*-methyl-2-pyrrolidone (135 ml), and Br-butyric-cholesterol (100 mg) dissolved in *N*-methyl-2-pyrrolidone was added (39 ml). The reaction was kept under magnetic stirring for 40 h, at 38 °C. Thereafter, an exhaustive dialysis against distilled water (Visking tubing, 12–14 KDa cut-off) was carried out, and the gellan-cholesterol was recovered by freeze-drying. The derivatization degree of gellan-cholesterol was 9% (mol of cholesterol *vs.* mol of carboxylic groups of the polymer) [5]. To produce the nanohydrogels, gellan-cholesterol (20 mg) was dispersed in PBS (10 ml) and the suspension was: (a) sonicated for 200 cycles, 5 s on / 2 s off, at 13 microns of probe amplitude, with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, UK) to obtain the sonicated (S) nanohydrogel; (b) autoclaved at 121 °C for 30 min to produce the autoclaved (A) nanohydrogel. Baicalin was added (10 mg/ml) to the pre-formed nanohydrogels and moderately sonicated (25 cycles, 2 s on / 2 s off), so that the polyphenol is homogeneously embedded within the nanohydrogel network.

2.3. Nanohydrogel characterization

Particle average diameter and polydispersity index (PI; a dimensionless measure of the broadness of the size distribution) were determined by Photon Correlation Spectroscopy using a Zetasizer nano-ZS (Malvern Instruments, Worcestershire, UK), which analyzes the fluctuations in intensity of the light backscattered by the particles in dispersion. Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures particle electrophoretic mobility [13].

Entrapment efficiency (EE%) was expressed as the percentage of baicalin recovered after dialysis *vs.* the amount initially used. Baicalin nanohydrogels (2 ml) were dialysed in Spectra/Por tubing (12–14 KDa cut-off) against PBS (2 l) for 2 h, to remove free baicalin. The nanoparticles were treated with methanol (1:1000) and the baicalin content was determined by high performance liquid chromatography (HPLC) using a Perkin Elmer Series 200 HPLC equipped with a UV detector and a column Teknokroma Brisa “LC2” C18 (5.0 μm, 150 × 4.6 mm). The mobile phase consisted of a

mixture of water and methanol (30:70), delivered at a flow rate of 1 ml/min. Baicalin content was measured at 278 nm [14].

2.4. Rheological characterization

Rheological measurements were carried out at 25±1 °C, using a Haake RheoStress 300 Rotational Rheometer, equipped with a Haake DC10 thermostat and data acquisition and elaboration software RheoWin. A cone-plate device (Haake CP35 TI diameter =35 mm) was used. Flow curves were evaluated in a shear rate range of 10^{-3}-10^{3} s^{-1}: a stepwise increase of the stress was applied, with an equilibration time of 30 s. All the measurements were carried out in triplicate, at 25 °C [15,16].

2.5. *In vitro* skin delivery studies

Non-occlusive experiments were performed using vertical Franz diffusion cells (0.785 cm^2 diffusion area) and newborn pig skin. One day-old Goland-Pietrain hybrid pigs (~1.2 kg) were provided by a local slaughterhouse. The excised skin was pre-equilibrated in saline at 37°C for 12 h, and then sandwiched between donor and receptor compartments of the Franz cells. The receptor was filled with saline (~6.5 ml), continuously stirred and thermostated at 37±1 °C. Samples (100 µl containing baicalin 10 mg/ml) were applied onto the skin specimens (n=6). Every 2 h and up to 8 h, the receiving solution was withdrawn, refreshed, and analysed by HPLC for baicalin content. After 8 h, the skin surface was gently washed with 1 ml of distilled water, and blotted on filter paper. The stratum corneum was removed by stripping with adhesive tape Tesa® AG (Hamburg, Germany). Epidermis was separated from dermis with a surgical scalpel. The tape strips and the skin strata were cut, placed each in a flask with methanol, and sonicated for 2 min in an ice bath to extract baicalin. The tape and tissue suspensions were filtered out and assayed for baicalin content by HPLC (see paragraph 2.3).

2.6. *In vitro* cell viability and protection against oxidative stress

3T3 fibroblasts were grown as monolayer in 75 cm^2 flasks incubated at 37 °C in humidified atmosphere of 5% CO_2. Dulbecco's Modified Eagle Medium high glucose containing L-glutamine,

and supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and 1% fungizone, was used as growth medium.

For the biocompatibility assay, the cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well, and after 24 h of incubation they were exposed to different concentrations of baicalin in PBS or in the nanohydrogels (20, 10, 1, 0.1 μg/ml) for 24 and 48 h. The empty nanohydrogels were tested for the appropriate comparison.

Cell viability was assessed by the MTT [3(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] colorimetric assay, by adding 100 μl of MTT reagent (0.5 mg/ml in PBS) to each well. After 3 h, the formed formazan crystals were dissolved in dimethyl sulfoxide, and the concentration was spectrophotometrically determined at 570 nm with a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). The results are reported as the percentage of untreated cells (100% viability).

For the antioxidant assay, the cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well. After 24 h of incubation, cells were exposed to hydrogen peroxide (1:50000 dilution in PBS) for 4 h, in the presence of baicalin in PBS, or baicalin embedded in the nanohydrogel (final concentration of baicalin: 20, 10, 1, 0.1 μg/ml). Cells treated with hydrogen peroxide-only were used as a positive control. After 4 h of exposure, the cells were washed with fresh medium, and their viability was determined by the MTT assay, as above.

2.7. *In vivo* topical application of baicalin nanohydrogels on TPA injured skin

Female CD-1 mice (5-6 weeks old, 25-35 g) were obtained from Envigo RMS S.r.l. (San Pietro al Natisone, Italy). The experiments were performed in accordance with the European Union regulations for the handling and use of laboratory animals, and the protocols were approved by the Institutional animal care and use committee of the University of Valencia (code A1456917886577). The back skin of mice was shaved one day before the experiment. On day 1, cutaneous inflammation/injury was induced by applying a phorbol ester (12-O-tetradecanoylphorbol 13-

acetate, TPA; 20 µl) dissolved in acetone (243 µM) on the shaved area (~2 cm^2), and after 3 h, baicalin in PBS or in the nanohydrogels, or Betnovate®, a commercial betamethasone steroid cream, were topically smeared (20 µl each) over the same site. Untreated mice were used as a negative control, and mice treated with TPA and PBS were used as a positive control. The procedure was repeated on day 2 and 3. On day 4, mice were sacrificed by cervical dislocation, and the treated skin area was excised, weighed, and immediately stored at −80 °C.

Oedema was measured as a function of the weight of the excised skin. The myeloperoxidase (MPO) activity was measured as previously reported [10]. Briefly, skin biopsies were homogenized and centrifuged. The supernatant was incubated with hydrogen peroxide and tetramethylbenzidine, and then assayed for MPO activity spectrophotometrically at 620 nm. The MPO activity was calculated from the linear portion of a standard curve. MPO and oedema inhibition were calculated as the percentage of values from treated animals *vs.* the positive control (TPA only-treated animals).

In addition, the skin specimens were homogenized in 50 nM phosphate buffer pH 7.4 containing protease inhibitors. Tumour necrosis factor-α (TNF-α) was assayed by using a kit from R&D Systems (Minneapolis, MN, USA) with a sensitivity of 5.0 pg/ml. Data were expressed as the percentage of inhibition of the cytokine found in the skin treated with the formulations *vs.* the amount found in the skin injured by TPA (positive control).

2.8. Statistical analysis of data

Results are expressed as the mean ± standard deviation. Analysis of variance (ANOVA test) was performed using IBM SPSS statistics for Windows. *Post hoc* testing ($P<0.05$) of the multiple comparisons was performed by the Scheffe's or Dunnet's test.

3. RESULTS

3.1. Nanohydrogel preparation and characterization

Aiming at optimizing the formulation of the nanohydrogels for the delivery of baicalin, the cholesterol-derivatized gellan was sonicated or autoclaved to induce the assembling of the polymer

chains in stable nanosized particles. Then, baicalin was added to the pre-formed nanohydrogels. The macroscopic appearance of the two nanohydrogels was very different: the sonicated one appeared as a yellow-transparent dispersion with some aggregated polymer particles adhering to the walls of the vial, while the autoclaved nanohydrogel appeared as a homogenous, yellow-transparent dispersion (Figure 1).

The sonication process was not able to produce a nanohydrogel with small nanoparticles: the average size was > 500 nm, and the PI was > 0.4 (Table 1). On the contrary, as already described in the literature [17], the autoclave method was demonstrated to be more efficient, as the nanoparticles of the autoclaved nanohydrogel were smaller and more homogenously dispersed (~350 nm, PI ≤0.30; Table 1), in accordance with the results of the visual observation. The zeta potential was sufficiently negative to ensure the stability of the nanohydrogels upon storage, thanks to the superficial electrostatic repulsion of the nanoparticles. The entrapment efficiency was approximately 37% for both nanohydrogels (Table 1).

The flow curves of sonicated and autoclaved nanohydrogels were measured (Figure 2). In agreement with the size results, the sonicated nanohydrogel, which had shown larger particle diameter, displayed higher viscosity, indicating a reduction of the interparticle free water and the formation of particle-particle interactions. On the contrary, in the autoclaved nanohydrogel, which had shown smaller particle size, the amount of free water in the interparticle domains was higher, thus resulting in lower viscosity values.

3.2. *In vitro* skin delivery of baicalin

In vitro skin delivery studies were carried out on newborn pig skin, and the amount of baicalin accumulated in the different skin layers and recovered in the receptor fluid was expressed as a percentage of the amount of baicalin applied on the skin surface (Figure 3). Results showed a similar baicalin accumulation in the stratum corneum and dermis for all the tested formulations (~1%, $p>0.05$). On the contrary, both nanohydrogels led to a deposited amount of baicalin in the

epidermis (~1%) significantly higher than that found using the polyphenol in PBS (~0.2%, $p<0.05$ *vs.* nanohydrogels). The amount of baicalin permeated into the receptor fluid was very low (~0.1%, $p>0.05$ among samples), irrespective of the formulation used.

3.3. *In vitro* cell viability and protection against oxidative stress

The biocompatibility of baicalin in PBS or embedded in nanohydrogels was assessed by using 3T3 fibroblasts (Figure 4). The cells were incubated with the baicalin formulations using different sample dilutions (1:500, 1:1000, 1:10000, 1:100000 corresponding to 20, 10, 1, 0.1 μg/ml of baicalin) for 24 and 48 h. After 24 and 48 h of exposure to baicalin in PBS, the viability of the cells was high (approximately 110%). A similar behaviour was observed after a 24h exposure to baicalin embedded in nanohydrogels, at all the dilution tested. After 48h, the viability of fibroblasts slightly decreased, but it was still around 100%, thus indicating a high biocompatibility of the nanohydrogels as well.

In consideration of the well-known antioxidant properties of baicalin, the ability of the formulations to prevent the oxidative damage generated by hydrogen peroxide in 3T3 cell culture was evaluated (Figure 5). Hydrogen peroxide is a toxic oxidant for cells as it causes high mortality, corresponding to approximately 50% under the current experimental conditions. The simultaneous treatment of the cells with the oxidant and baicalin was able to counteract the oxidative effect of hydrogen peroxide, restoring normal conditions (~95% viability), regardless of the applied dose (20, 10, 1 and 0.1 μg/ml) and the formulation used (baicalin in PBS or embedded in nanohydrogels). These results confirmed the actual cytoprotective effect of this natural polyphenol, irrespective of its inclusion in the nanohydrogels.

3.4. *In vivo* wound healing potential of baicalin

The back skin of mice was injured by topically applied TPA, and the lesion was treated with the baicalin in PBS or embedded in nanohydrogels. The anti-inflammatory and repair activities of

baicalin and their enhancement by incorporation into transfersomes have been previously demonstrated [10]: results reported therein showed that transfersomes loaded with baicalin at different concentrations (2.5, 5 and 10 mg/ml) healed TPA lesions and inhibited markers of inflammation (i.e. MPO) more efficiently than dexamethasone solution (a potent anti-inflammatory drug) and baicalin in PBS (10 mg/ml).

In the present work, given the anti-inflammatory activity of baicalin, the efficacy of nanohydrogels was assessed and compared with that of Betnovate®, a commercial cream containing anti-inflammatory betamethasone. Inflammation, which represents a part of the acute response to tissue injury, involves a coordinated influx of neutrophils that produce and release inflammatory mediators, and high levels of destructive proteases and oxygen free radicals. This can cause extensive tissue damage and prolong the inflammatory phase. Non-phagocytic cells also generate free radicals, leading to an accumulation of oxygen and nitrogen reactive species at the wound site. One of the major causes of delayed healing is the persistence of inflammation or an inadequate angiogenic response. It has been postulated that an anti-inflammatory response after cutaneous wounding is a prerequisite for healing. Potent antioxidant and anti-inflammatory agents can play an important role in restoring physiological conditions, allowing a significant improvement in wound healing. Their delivery at the wound site by innovative vesicles may improve their residence time, and favour their up-take by keratinocytes and fibroblasts.

The macroscopic evaluation of the mice skin was performed at the end of the treatment by visual inspection (Figure 6). The skin treated with TPA only (positive control) appeared markedly damaged, with loss of epidermis, especially in the peripheral area, and a thin crust [18]. The wound formation was partially reduced by the betamethasone cream, as well as by baicalin in PBS, while it was strongly prevented by using the baicalin embedded in autoclaved nanohydrogel: the skin texture was comparable to that of the negative control (healthy untreated skin).

Additionally, the inhibition (expressed as inhibition percentage) of inflammation markers, such as oedema, MPO, and TNF-α was measured (Table 2). The treatment with betamethasone cream did not inhibit effectively the studied markers, as the obtained results were not statistically different from the positive control ($p>0.05$). The oedema and MPO inhibition provided by the baicalin autoclaved (A) nanohydrogel was the highest (78% and 44%, respectively; $p<0.05$ *vs.* positive control, betamethasone cream and baicalin in PBS), confirming the importance of the formulation in promoting baicalin efficacy on damaged skin. Additionally, the cytokine inhibition provided by baicalin autoclaved nanohydrogel was ~100% ($p<0.05$ *vs.* positive control, betamethasone cream and baicalin in PBS), which was the highest value obtained among the different formulations.

Discussion

Wounds are physical injuries characterized by an opening or breaking of the skin. This kind of injury is difficult to repair, and involves a set of pathological events. For the restoration of disrupted anatomical continuity and altered functional status of the skin, a proper treatment that delivers the drug to the deeper skin layers where pathological process occurs, is needed. Previous studies demonstrated that baicalin possesses stronger antioxidant activity than ascorbic acid and butylated hydroxytoluene [19]. Nanohydrogels have shown promising effects in the topical treatment of skin diseases, as a consequence of their ability to incorporate high volumes of water, thus acting as moisturizing agents and enhancing stratum corneum hydration [20]. Therefore, thanks to their favourable features (biocompatibility, hydration properties and nanosize), in the present work nanohydrogels were investigated as topical delivery systems capable of enhancing baicalin skin delivery and efficacy. The radical-scavenging capability of baicalin was assessed against the free radicals generated by hydrogen peroxide in cells, and the effect of the nanohydrogel carrier on the baicalin antioxidant activity was evaluated.

The nanohydrogels were prepared by sonicating or autoclaving a gellan-cholesterol derivative: the latter was demonstrated to be the most efficient process, as it produced smaller and more

homogeneously dispersed nanoparticles, which resulted in less viscous system due to a higher amount of free water in the interparticle domains [17]. Autoclaving and sonication, due to their different physico-chemical interactions with the polymer chains and the aqueous environment, led to the production of different nanohydrogel systems. In particular, we assume that they cause a different assembling of the polymer chains, which resulted in different particle features, especially in terms of size and homogeneity.

The physico-chemical and technological properties of the prepared systems seem to indicate that the autoclaved (A) nanohydrogel is more suitable for baicalin delivery, because of its higher homogeneity (due to the particles' features) and viscosity, as compared to baicalin in PBS, thus, ensuring a more effective spreadability of the formulation on the skin.

Despite their different physico-chemical properties, the two nanohydrogels showed comparable performances *in vitro*, as they provided a similar accumulation of baicalin into the deeper skin layers. Both baicalin in PBS and embedded in the nanohydrogels were highly biocompatible and capable of protecting skin cells from oxidative stress caused by hydrogen peroxide by reducing cell mortality, thus confirming that the activity of the polyphenol in cells was not affected by the nanocarrier.

Since the two nanohydrogel formulations showed similar performances *in vitro*, the *in vivo* studies were carried out using the autoclaved (A) nanohydrogel, by virtue of the abovereported best technological features (i.e., size, homogeneity, and viscosity). To evaluate the effect of the delivery system, the results were compared with those of baicalin in PBS and a commercial cream containing betamethasone, a potent steroidal anti-inflammatory drug. The topical administration of the baicalin autoclaved nanohydrogel onto TPA-injured mouse skin led to complete wound healing, as indicated by visual observation. In addition, from a quantitative point of view, this formulation led to a complete inhibition of TNF-α, and almost complete inhibition of oedema. The nanohydrogel showed optimal performances as skin carriers. Probably, thanks to its hydrophilic

nature, the nanohydrogel acts as moisturizing agent on the stratum corneum, leading to the accumulation of baicalin in the epidermis, followed by its diffusion into the dermis where it can counteract oxidative stress, regulating the inflammation process and promoting skin repair.

Conclusions

Gellan-cholesterol nanohydrogels were produced by using two different methods: sonication and autoclaving. The autoclaved nanohydrogel seems to be the most promising carrier in terms of size, homogeneity, and rheological properties. The suitability of both nanohydrogels as carriers for baicalin skin delivery was evaluated, and the superior performances in comparison with baicalin in PBS were demonstrated. Further, the autoclaved nanohydrogel showed optimal performances in the topical treatment of skin wound, and might be proposed as an effective formulation against skin diseases related to inflammation and oxidative stress.

Acknowledgements

Financial support from University "Sapienza": "Finanziamenti di Ateneo per la Ricerca Scientifica-Anno 2016", and Progetto di Ricerca RM11715C1743EE89 are acknowledged.

References

[1] Y. Fan, J. Yi, X. Hua, Y. Zhang, R. Yang, Preparation and characterization of gellan gum microspheres containing a cold-adapted β-galactosidase from Rahnella sp. R3, Carbohydr. Polym. 162 (2017) 10–15. doi:10.1016/j.carbpol.2017.01.033.

[2] C.S.F. Picone, R.L. Cunha, Influence of pH on formation and properties of gellan gels, Carbohydr. Polym. 84 (2011) 662–668. doi:10.1016/j.carbpol.2010.12.045.

[3] S.A. Agnihotri, S.S. Jawalkar, T.M. Aminabhavi, Controlled release of cephalexin through gellan gum beads: Effect of formulation parameters on entrapment efficiency, size, and drug release, Eur. J. Pharm. Biopharm. 63 (2006) 249–261. doi:10.1016/j.ejpb.2005.12.008.

[4] G. D'Arrigo, G. Navarro, C. Di Meo, P. Matricardi, V. Torchilin, Gellan gum nanohydrogel containing anti-inflammatory and anti-cancer drugs: a multi-drug delivery system for a

combination therapy in cancer treatment, Eur. J. Pharm. Biopharm. 87 (2014) 208–216. doi:10.1016/j.ejpb.2013.11.001.

[5] G. D'Arrigo, C. Di Meo, E. Gaucci, S. Chichiarelli, T. Coviello, D. Capitani, F. Alhaique, P. Matricardi, Self-assembled gellan-based nanohydrogels as a tool for prednisolone delivery, Soft Matter. 8 (2012) 11557. doi:10.1039/c2sm26178b.

[6] N. Zoratto, F. Palmieri, C. Cencetti, E. Montanari, C. Di Meo, M. Manca, M. Manconi, P. Matricardi, Design of Hybrid Gels Based on Gellan-Cholesterol Derivative and P90G Liposomes for Drug Depot Applications, Gels. 3 (2017) 18. doi:10.3390/gels3020018.

[7] W. Lee, S.K. Ku, J.S. Bae, Anti-inflammatory Effects of Baicalin, Baicalein, and Wogonin In Vitro and In Vivo, Inflammation. 38 (2014) 110–125. doi:10.1007/s10753-014-0013-0.

[8] Y.-C. Shen, W.-F. Chiou, Y.-C. Chou, C.-F. Chen, Mechanisms in mediating the anti-inflammatory effects of baicalin and baicalein in human leukocytes, Eur. J. Pharmacol. 465 (2003) 171–181. doi:10.1016/S0014-2999(03)01378-5.

[9] P.-L. Tsai, T.-H. Tsai, Pharmacokinetics of Baicalin in Rats and its Interactions with Cyclosporin A, Quinidine and SKF-525A: A Microdialysis Study, Planta Med. 70 (2004) 1069–1074. doi:10.1055/s-2004-832649.

[10] S. Mir-Palomo, A. Nácher, O. Díez-Sales, M.A. Ofelia Vila Busó, C. Caddeo, M.L. Manca, M. Manconi, A.M. Fadda, A.R. Saurí, Inhibition of skin inflammation by baicalin ultradeformable vesicles, Int. J. Pharm. 511 (2016) 23–29. doi:10.1016/j.ijpharm.2016.06.136.

[11] M. Manconi, M.L. Manca, C. Caddeo, D. Valenti, C. Cencetti, O. Diez-Sales, A. Nacher, S. Mir-Palomo, M.C. Terencio, D. Demurtas, J.C. Gomez-Fernandez, F.J. Aranda, A.M. Fadda, P. Matricardi, Nanodesign of new self-assembling core-shell gellan-transfersomes loading baicalin and in vivo evaluation of repair response in skin, Nanomedicine Nanotechnology, Biol. Med. (n.d.) In press 2017.

[12] M. Manconi, M.L. Manca, C. Caddeo, D. Valenti, C. Cencetti, O. Diez-Sales, A. Nacher, S. Mir-Palomo, M.C. Terencio, D. Demurtas, J.C. Gomez-Fernandez, F.J. Aranda, A.M. Fadda, P. Matricardi, Nanodesign of new self-assembling core-shell gellan-transfersomes loading baicalin and in vivo evaluation of repair response in skin, Nanomedicine Nanotechnology, Biol. Med. 14 (2018) 569–579. doi:10.1016/J.NANO.2017.12.001.

[13] M.L. Manca, I. Castangia, C. Caddeo, D. Pando, E. Escribano, D. Valenti, S. Lampis, M. Zaru, A.M. Fadda, M. Manconi, Improvement of quercetin protective effect against oxidative stress skin damages by incorporation in nanovesicles., Colloids Surf. B. Biointerfaces. 123 (2014) 566–574. doi:10.1016/j.colsurfb.2014.09.059.

[14] M.L. Manca, M. Manconi, D. Valenti, F. Lai, G. Loy, P. Matricardi, A.M. Fadda, Liposomes coated with chitosan-xanthan gum (chitosomes) as potential carriers for pulmonary delivery of rifampicin, J. Pharm. Sci. 101 (2011) 566–575. http://www.scopus.com/inward/record.url?eid=2-s2.0-84455169626&partnerID=40&md5=5a747c39be021f92e38cbeead2c5b19c.

[15] M.L. Manca, M. Manconi, A.M. Falchi, I. Castangia, D. Valenti, S. Lampis, A.M. Fadda, Close-packed vesicles for diclofenac skin delivery and fibroblast targeting, Colloids Surfaces B Biointerfaces. 111 (2013) 609–617. doi:http://dx.doi.org/10.1016/j.colsurfb.2013.07.014.

[16] M.L. Manca, I. Castangia, P. Matricardi, S. Lampis, X. Fernàndez-Busquets, A.M. Fadda, M. Manconi, Molecular arrangements and interconnected bilayer formation induced by alcohol or polyalcohol in phospholipid vesicles., Colloids Surf. B. Biointerfaces. 117 (2014) 360–367. doi:10.1016/j.colsurfb.2014.03.010.

[17] E. Montanari, C. Di Meo, S. Sennato, A. Francioso, A.L. Marinelli, F. Ranzo, S. Schippa, T. Coviello, F. Bordi, P. Matricardi, Hyaluronan-cholesterol nanohydrogels: Characterisation and effectiveness in carrying alginate lyase, N. Biotechnol. 37 (2017) 80–89. doi:10.1016/j.nbt.2016.08.004.

[18] C. Caddeo, A. Nacher, A. Vassallo, M.F. Armentano, R. Pons, X. Fernàndez-Busquets, C. Carbone, D. Valenti, A.M. Fadda, M. Manconi, Effect of quercetin and resveratrol co-incorporated in liposomes against inflammatory/oxidative response associated with skin cancer, Int. J. Pharm. 513 (2016) 153–163. doi:10.1016/j.ijpharm.2016.09.014.

[19] L. Peng-fei, H. Fu-gen, D. Bin-bin, D. Tian-sheng, H. Xiang-lin, Z. Ming-qin, Purification and antioxidant activities of baicalin isolated from the root of huangqin (Scutellaria baicalensis gcorsi), J. Food Sci. Technol. 50 (2013) 615–619. doi:10.1007/s13197-012-0857-y.

[20] F. Rancan, M. Asadian-Birjand, S. Dogan, C. Graf, L. Cuellar, S. Lommatzsch, U. Blume-Peytavi, M. Calderón, A. Vogt, Effects of thermoresponsivity and softness on skin penetration and cellular uptake of polyglycerol-based nanogels, J. Control. Release. 228 (2016) 159–169. doi:10.1016/j.jconrel.2016.02.047.

Figure Captions

Figure 1. Macroscopic appearance of baicalin sonicated (S) and autoclaved (A) nanohydrogels.

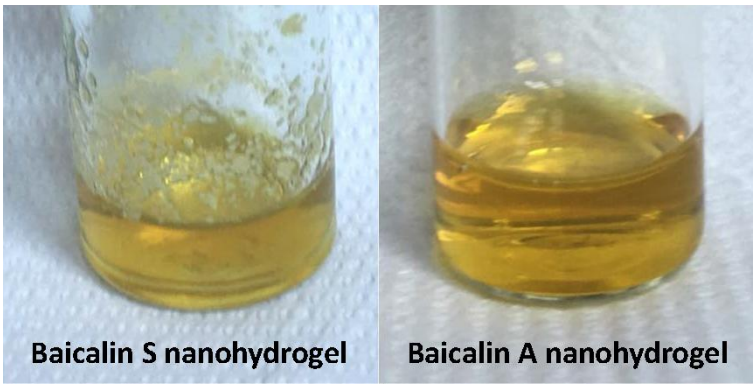
Figure 2. Representative flow curves of baicalin sonicated (S) and autoclaved (A) nanohydrogels.

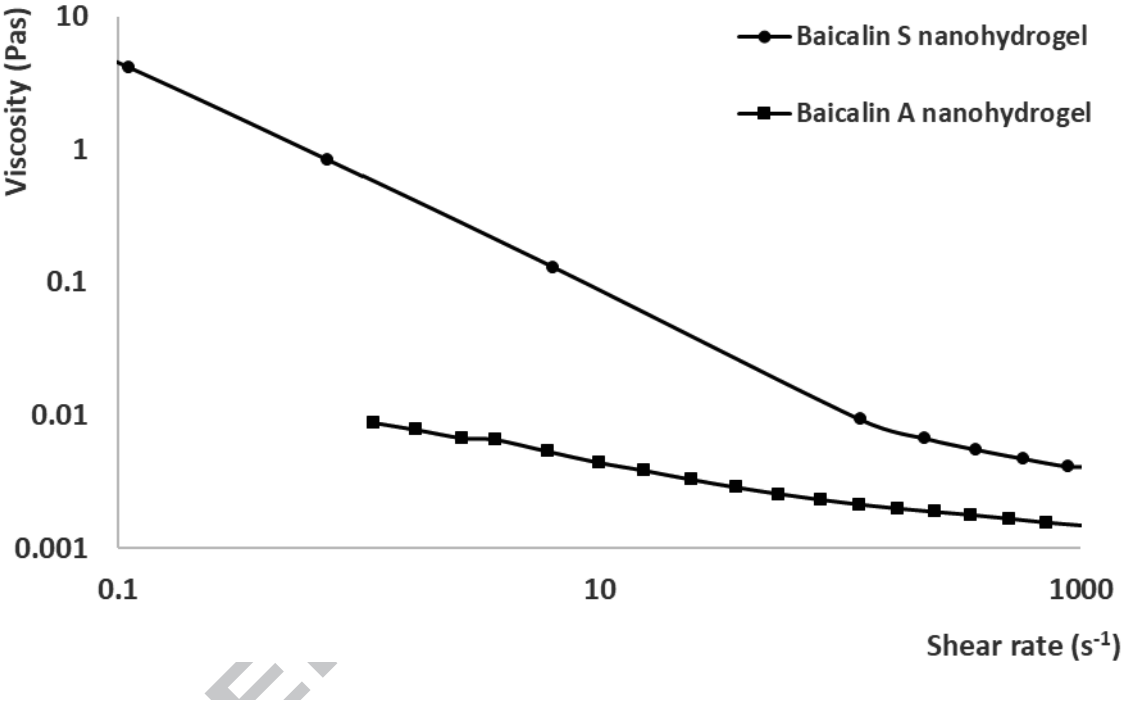
Figure 3. Baicalin accumulated in the stratum corneum (SC), epidermis (EP) dermis (D) and receptor fluid (RF) after application of baicalin in PBS or in sonicated (S) or autoclaved (A) nanohydrogels after 8 h. Mean values ± standard deviation (error bars) are reported.

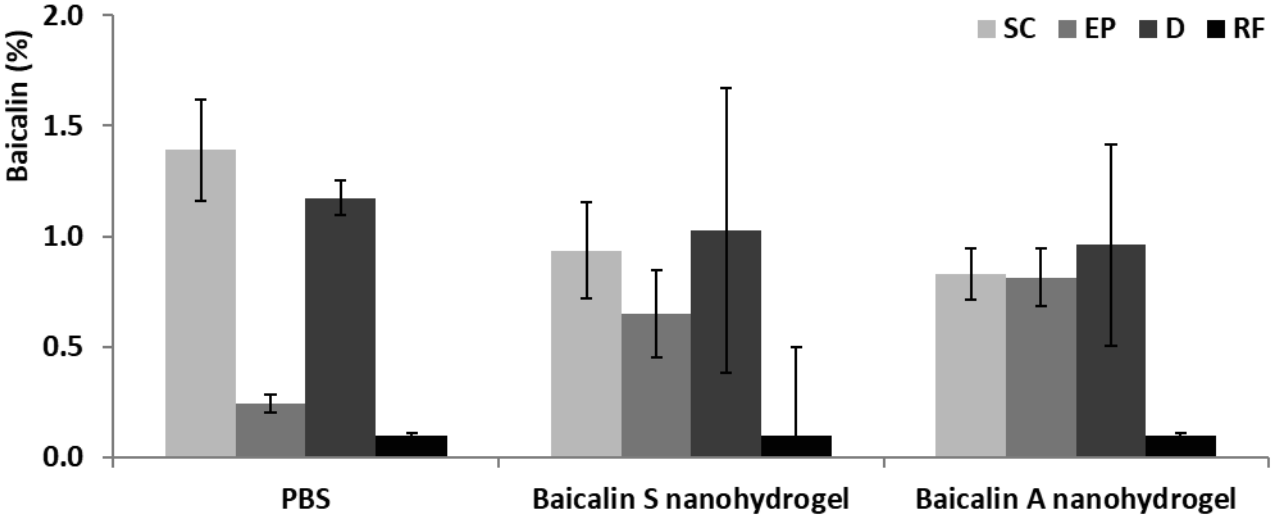
Figure 4. *In vitro* viability of 3T3 fibroblasts incubated for 24 h (A) and 48 h (B) with different sample dilutions corresponding to 20, 10, 1 and 0.1 μg/ml of baicalin in PBS or in sonicated (S) or autoclaved (A) nanohydrogels. Data are reported as mean values ± standard deviation of cell viability expressed as the percentage of untreated cells (100% viability).

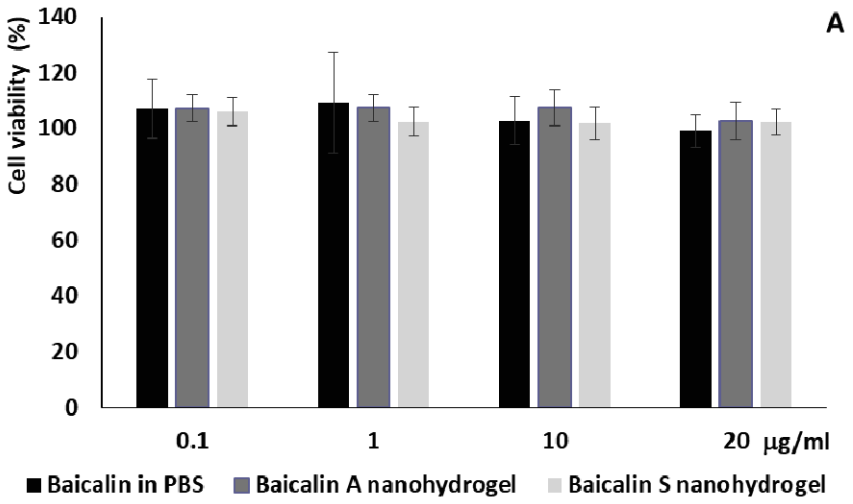
Figure 5. *In vitro* viability of 3T3 fibroblasts treated for 4 h with hydrogen peroxide and different sample dilutions corresponding to 20, 10, 1 and 0.1 μg/ml of baicalin in PBS or in sonicated (S) or autoclaved (A) nanohydrogels. Data are reported as the mean values ± standard deviation of cell viability expressed as the percentage of untreated cells (100% viability).

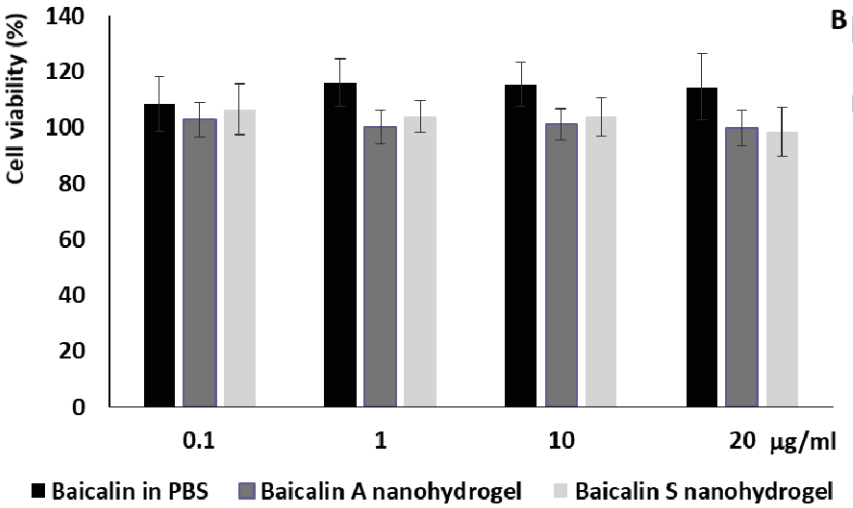
Figure 6. Back skin of mice treated with PBS only (negative control) or exposed to TPA and treated with PBS (positive control), or betamethasone commercial cream (Betnovate®), baicalin in PBS, baicalin in autoclaved (A) nanohydrogel.

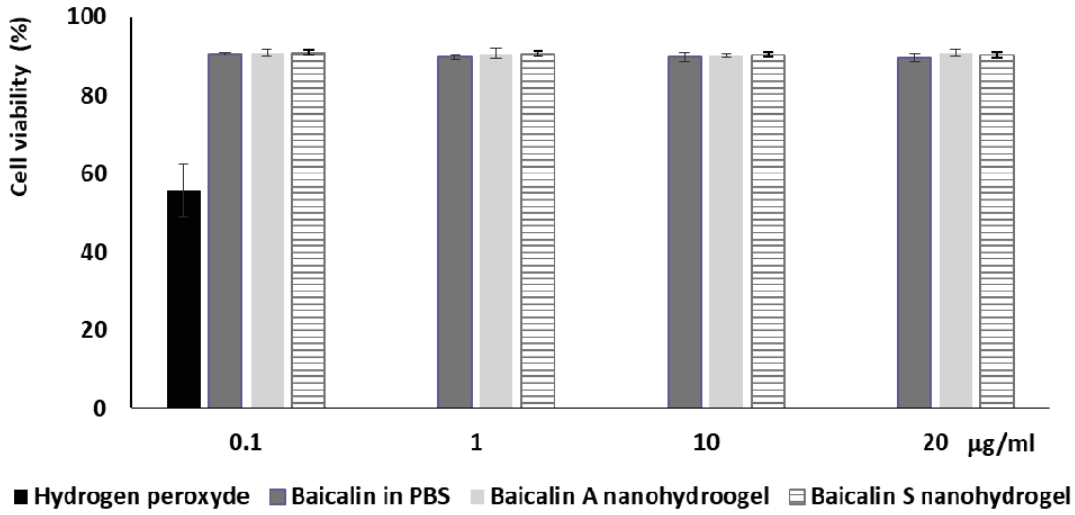

Baicalin S nanohydrogel
Baicalin A nanohydrogel


Viscosity (Pas)
10
1
0.1
0.01
0.001
0.1
10
1000
Shear rate (s-1)
Baicalin S nanohydrogel
Baicalin A nanohydrogel


Baicalin (%)
2.0
1.5
1.0
0.5
0.0
SC
EP
D
RF
PBS
Baicalin S nanohydrogel
Baicalin A nanohydrogel


A
Cell viability (%)
140
120
100
80
60
40
20
0
0.1
1
10
20 μg/ml
Baicalin in PBS
Baicalin A nanohydrogel
Baicalin S nanohydrogel


B
Cell viability (%)
140
120
100
80
60
40
20
0
0.1
1
10
20 μg/ml
Baicalin in PBS
Baicalin A nanohydrogel
Baicalin S nanohydrogel


Cell viability (%)
100
80
60
40
20
0
0.1
1
10
20 μg/ml
Hydrogen peroxyde
Baicalin in PBS
Baicalin A nanohydroogel
Baicalin S nanohydrogel

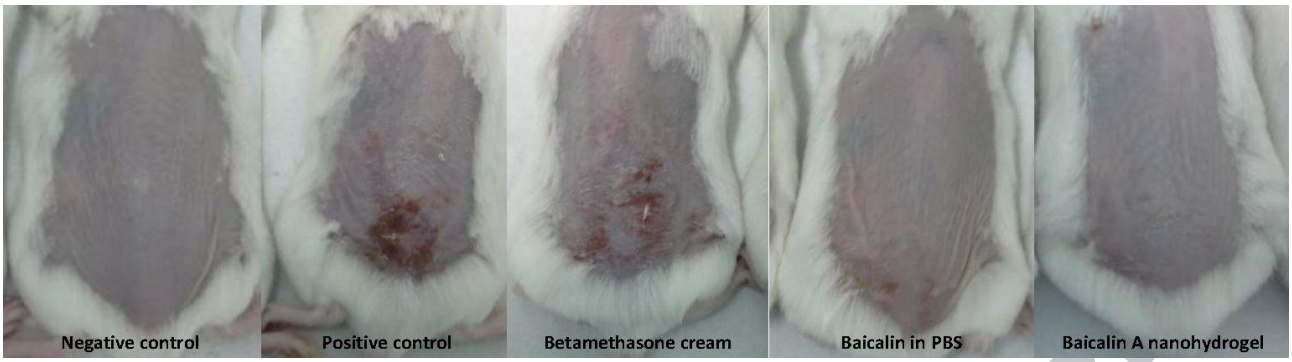


Table 1. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of empty and baicalin sonicated (S) or autoclaved (A) nanohydrogels. Each value represents the mean ± standard deviation of at least six determinations.

	MD (nm)	**PI**	**ZP (mV)**	**EE (%)**
Empty S nanohydrogel	554±61	0.45	-30±7	
Empty A nanohydrogel	383±23	0.19	-24±2	
Baicalin S nanohydrogel	507±68	0.49	-25±3	38±9
Baicalin A nanohydrogel	341±45	0.30	-23±4	35±9

Table 2. Inhibition of oedema, MPO and TNF-α in mice exposed to TPA and treated with PBS (positive control), or betamethasone commercial cream, baicalin in PBS or in autoclaved (A) nanohydrogel. Mean values ± standard deviation are reported. $^{*,\ \circ,\ +}$ $p < 0.05\%$ *vs.* the other groups.

	Oedema I (%)	**MPO I (%)**	**TNF-α I (%)**
Positive control	0	0	0
Betamethasone cream	43±28	0	0
Baicalin in PBS	25±20	23±18	4±3
Baicalin A nanohydrogel	*78±15	°44±25	$^{+}$100±26

Graphical abstract