Colloids and Surfaces B: Biointerfaces 234 (2024) 113678

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Colloidal hydrogel systems of thymol-loaded PLGA nanoparticles designed for acne treatment

Camila Folle [a,*], Ana M. Marqués [b], Mireia Mallandrich [a,c], Joaquim Suñer-Carbó [a,c], Lyda Halbaut [a,c], Elena Sánchez-López [a,c,**], Ana Laura López-Machado [a,c], Natalia Díaz-Garrido [d,e,f], Josefa Badia [d,e,f], Laura Baldoma [d,e,f], Marta Espina [a,c], María Luisa García [a,c], Ana Cristina Calpena [a,c]

[a] Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona 08028, Spain
[b] Department of Biology, Healthcare and Environment, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona 08028, Spain
[c] Institute of Nanoscience and Nanotechnology (IN2UB), University of Barcelona, Barcelona 08028, Spain
[d] Department of Biochemistry and Physiology, Biochemistry and Biomolecular Science, University of Barcelona, Barcelona 08028, Spain
[e] Institute of Biomedicine of the University of Barcelona (IBUB), Barcelona 08028, Spain
[f] Research Institute Sant Joan de Déu (IR-SJD), Barcelona 08950, Spain

ARTICLE INFO

Keywords:
Thymol
PLGA nanoparticles
Colloidal gels
Skin permeation
Skin microbiota
Acne treatment

ABSTRACT

Thymol-loaded PLGA nanoparticles (TH-NPs) were incorporated into different semi-solid formulations using variable gelling agents (carbomer, polysaccharide and poloxamer). The formulations were physicochemically characterized in terms of size, polydispersity index and zeta potential. Moreover, stability studies were performed by analyzing the backscattering profile showing that the gels were able to increase the nanoparticles stability at 4 °C. Moreover, rheological properties showed that all gels were able to increase the viscosity of TH-NPs with the carbomer gels showing the highest values. Moreover, the observation of carbomer dispersed TH-NPs under electron microscopical techniques showed 3D nanometric cross-linked filaments with the NPs found embedded in the threads. In addition, cytotoxicity studies showed that keratinocyte cells in contact with the formulations obtained cell viability values higher than 70 %. Furthermore, antimicrobial efficacy was assessed against *C. acnes* and *S. epidermidis* showing that the formulations eliminated the pathogenic *C. acnes* but preserved the resident *S. epidermidis* which contributes towards a healthy skin microbiota. Finally, biomechanical properties of TH-NPs dispersed in carbomer gels in contact with healthy human skin were studied showing that they did not alter skin properties and were able to reduce sebum which is increased in acne vulgaris. As a conclusion, TH-NPs dispersed in semi-solid formulations and, especially in carbomer gels, may constitute a suitable solution for the treatment of *acne vulgaris*.

1. Introduction

Acne vulgaris is one of the most common dermal inflammatory disorders treated by health care specialists [1]. Although it peaks around 17 years, adult acne is also fairly common, especially in women, with an incidence between 5 % and 12 % [2]. It is a multifactorial pathology of the pilosebaceous unit associated with an imbalance in skin microbiota and hyperactivity of the sebaceous glands. It produces elevated levels of sebum, hyperkeratosis by blockage of the hair follicle, and the etiopathogenic factors of excessive microbiota reproduction [3]. In sebum-rich sites such as scalp, face, chest and back, *C. acnes* forms up to 90 % of microbiota. It is a rod-shaped Gram-positive and aerotolerant anaerobic bacterium found as a normal resident of healthy skin. Although it is essential for sebum control and proper pH pilosebaceous follicle, it had been reported that this bacteria is likely to proliferate under unbalanced function of the sebaceous glands [4-6]. However, skin microbiota is

* Corresponding author.
** Corresponding author at: Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona 08028, Spain.
E-mail addresses: camilafolle@ub.edu (C. Folle), esanchezlopez@ub.edu (E. Sánchez-López).

https://doi.org/10.1016/j.colsurfb.2023.113678
Received 6 October 2023; Received in revised form 20 November 2023; Accepted 26 November 2023
Available online 1 December 2023

formed by a delicate balance between several microorganisms. Therefore, when *C. acnes* increases, it contributes to inflammation and acne development [7]. In healthy skin, *C. acnes* is in equilibrium with *Staphylococcus epidermidis,* which is the most abundant non-pathogenic bacterium of human skin. *S. epidermidis* may exert a probiotic function by preventing colonization of other pathogenic bacteria [8]. Therefore, when an unbalanced function of the sebaceous glands occurs, *C. acnes* proliferates, behaving as pathogenic and leading to acne development. Therefore, a dual activity of *C acnes* on the skin microbiota may be considered, being a non-pathogenic essential for sebum control, as well as active pathogenic on acne infection and inflammation.

The treatment recommendations for Acne vulgaris depend on the severity of the condition but high levels would require topical antibiotics and retinoids [9]. In addition to antibiotic resistance concerns, treatments are also associated with skin dryness and irritation reducing adherence and suitable outcomes. Moreover, antibiotics potentially damage the entire skin microbiome disrupting the microbiota balance, therefore the skin remains without defense to external pathogens, being prone to other disorders.

Therefore, effective treatments that do not disrupt the entire microbiota balance constitute an unmet medical need. Therefore, natural compounds such as thymol (TH) could be the key for preventing or ameliorating acne associated symptoms [10]. TH is a monoterpene that possess pharmacological activities such as antioxidant, antimicrobial, antifungal, antiseptic, as well as anti-inflammatory [11-13]. Previous studies [14] showed the capacity of TH to increase elasticity and porosity, which are essential on cicatrizing/healing processes. TH, used as preservative in cosmetics and food by its antimicrobial activity [15], is considered safe in cosmetic formulations up to 0.5 %, according to the Cosmetic Ingredients Review (CIR). Despite its suitable pharmaceutical activity, TH is also known to possess permeation enhancing properties, disturbing the lipids of the stratum corneum (SC) [16]. For this reason, TH may have difficultly to remain for long time inside the skin [17,18].

In this field, the encapsulation of actives such as TH into nanoscopic carriers may constitute a suitable strategy to overcome TH drawbacks, solubilize it and achieve a prolonged therapeutic efficacy. Among several carriers, biodegradable nanoparticles, based on polymeric PLGA matrix (NPs), constitute one of the most studied colloidal systems for several applications, improving compounds penetration [19-21]. These colloidal systems, with an average particle size ranged between 10 and 1000 nm, are able to increase drug bioavailability and reduce its toxicity [22,23].

Furthermore, in order to increase patient comfort and applicability of the formulation, achieving longer retention times, hydrogels have been postulated as a suitable strategy [24]. Among several molecules, polysaccharides such as hydroxypropyl methylcellulose (HPMC) and polyacrylic acid derivatives such as carbomers are commonly used for dermal application [25]. In this field, HPMC is a hydrophilic polymer with good gelling characteristics and strong swelling properties that can rapidly hydrate, spread and adhere to skin surfaces [26]. In addition, carbomer is a mucoadhesive polymer with strong in situ gelling capacity, ease of spreading and depending on the type of excipient formulated it exhibits either aqueous or creamy sensory profile [27]. Moreover, poloxamers have been also recently used for dermal applications. Poloxamers are non-toxic and non-irritant synthetic tri-block copolymers. They confer thermo-reversible properties to the formulation being able to increase the viscosity at high temperatures transforming into a semi-solid transparent gel [28].

In previous studies, our group has found out an outstanding potential of surface functionalized TH loaded PLGA nanoparticles (TH-NPs) for the treatment of *acne vulgaris*, demonstrating to be effective as antimicrobial, anti-inflammatory and wound healing [17,18]. However, topical application of liquid TH-NPs is not a feasible strategy. Therefore, using the previously developed TH-NPs, we have incorporated them into several semi-solid formulations and applied them in human volunteers. In the present study, several gelling agents were examined to disperse TH-NPs and their rheological, stability and physicochemical and morphometrical properties were evaluated and compared. Moreover, with these hydrogels, the in vitro and in vitro release studied were performed. In addition, antimicrobial therapeutic efficacy was confirmed and in vivo studies in human volunteers assessing the trans-epidermal water loss and sebum quantification were carried out.

2. Material and methods

2.1. Materials

Thymol (TH), polysorbate 20 (Tween® 20, TW), poloxamer 188 (Kolliphor®, PX), poloxamer 407 (Pluronic®F127, PLUR), propylene glycol (PG), glycerin (GLY) and hydroxypropyl methylcellulose (HPMC) were supplied by Sigma Aldrich (Madrid, Spain). Carbomer (Carbopol® 934, C934) was purchased from Fagron Iberica (Barcelona, Spain) and chitosan (CS) was obtained from HMC+ (GmbH, Saale, Germany). Phosphatidylcholine (PL) was acquired from Lipoid® (GmbH, Ludwigshafen am Rhein, Germany) and Transcutol® P was a gift from Gattefossé (Cedex, France). Milli-Q water was obtained from filtration in a Millipore system (Molsheim, France). All other chemicals and reagents were from analytical grade.

Culture media Mueller Hinton Broth (MHB), Brain Heart Infusion (BHI) Clostridium reinforced medium (CRM), Tryptone Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA) were acquired from Oxoid (Basingstoke, UK). For cell culture, DMEM (Dulbecco's Modified Eagle's Medium) was supplied by ThermoFisher, MTT (3-(4,5-Dimethylthiazol-2-yl)–2,5-diphenyl tetrazolium bromide) and DMSO (99 % dimethyl sulfoxide) were purchased from Sigma-Aldrich (Barcelona, Spain).

2.2. Preliminary formulation development

Prior to the preparation of the semi-solid formulations, TH-NPs were developed as described in previous studies [17,18]. Briefly, thymol-loaded PLGA nanoparticles (TH-NPs) were produced by solvent displacement evaporation method [29]. The stabilizing agents on the surface of the NPs were either TW (TH-NP-T-), PL (TH-NP-L-) and PX (TH-NP-P-), obtaining negatively charged particles, and additionally, CS was also added to obtain a positively charged (TH-NP-P-C+).

2.3. Preparation of TH-NPs gel formulations

The preparation of the gels consisted of the incorporation of TH or TH-NPs into the total water content of the final formulation, obtaining a final concentration of TH at 0.1–0.25 %. Additionally, 5 % of either GLY or PG were added to the water phase of the formulations. The semi-solid formulations were prepared by adding the gelling agents (C934 at 0.5 %, HPMC 3 % or PLUR 20 %) into the aqueous phase until complete dissolution, using Unguator® (Microcaya, Bilbao, Spain) emulsifier. For the carbomer gels (GC), the mixture was allowed to stand overnight at room temperature (RT), and the pH was adjusted to 5.0–5.5 or 6.0–6.5 with NaOH 2 N to obtain a slightly fluid or viscous gel. For the HPMC gels (GH), the viscous homogeneous formulation was obtained once the mixture was completely homogeneous at RT, then the pH was adjusted to 5.0–5.5 with HCl 0.1 M. For the pluronic gel (GP), the mixture was prepared with the water phase cooled down to 4 °C and mixed until complete homogeneity, then, allowed to stand overnight at 4 °C, the pH was adjusted to 5.0–5.5 with HCl 0.1 M, and the viscous gel was formed once placed at RT.

2.4. Characterization of TH-NPs gel formulations

The physicochemical properties of TH-NPs were analysed using a ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK), measuring the average particle size and polydispersity index (Z_{av} and PI, respectively) before and after incorporation into the gel matrix. For these

measurements, samples were previously diluted in Milli-Q water (1:100) under vigorous stirring using a vortex mixer.

2.5. Stability of GC-TH-NPs developed formulations

The physical stability of TH-NPs gels was studied using Turbiscan® Lab Expert (Iesmat, Madrid, Spain), an optical analyser based on the multiple light scattering, with an applied light source corresponding to a pulsed near infrared (λ = 880 nm) through the glass measurement cell containing the sample. The detector at 45° from the incident beam was used to measure the backscattering signal (BS %) [30].

The carbomer gel formulations corresponding to functionalized TH-NPs previously developed [18], were compared regarding their accelerated stability conditions, stored for 1 month at RT (23 ± 2), 30 and 40 °C, and at 4 °C extending the storage at up to 12 months. Additionally, the stability of selected gels was followed for 6 months stored at RT and 12 months at 4 °C. The evaluated parameters were Z_{av}, PI and/or only organoleptic properties and pH (pH meter GLP 22, Crison Instruments, Alella, Spain).

The microbial preservative activity stability of developed gels was evaluated after storage at RT and 4 °C for 6 and 12 months, respectively. The method used was applied following specifications described in the European Pharmacopeia monographs (2.6.12. Microbiological examination of non-sterile products: total viable aerobic count). For this, samples (0.1 g) were transferred into the culture plates (TSA for bacteria and SDA for fungi and yeast) by inclusion, and the total viable count was carried out after incubation at 35 ± 2 °C for 3 days or at 28 ± 2 °C for 7 days, for bacteria and fungi/yeast, respectively [17].

2.6. Rheological behavior

Rheological measurements of the several TH and TH-NPs gels were determined with a Haake Rheostress® 1 rheometer (Thermo Fisher Scientific, Karlsruhe, Germany) connected to a thermostatic circulator Thermo Haake Phoenix II + Haake C25P and a computer Pc provided with Haake RheoWin® Job Manager and Data Manager software v. 4.91. Steady-state measurements were addressed with a cone-and-plate geometry (C60/2°Ti: 60 mm diameter, 2 ° angle). The shear stress (τ) was measured as a function of the shear rate (γ). Viscosity curves (η = f(γ)) and flow curves (τ = f(γ)) were recorded at 25 ± 0.1 °C. The shear rate ramp program included 3 min ramp-up period from 0 to 100 s^{-1}, 1 min constant shear rate period at 100 s^{-1}, and 3 min ramp-down from 100 to 0 s^{-1}. Data from the flow curves were fitted using mathematical models to identify the model that provided the best overall match of the experimentally observed rheological data. The adequacy of the rheological profiles to the mathematical models was based on the correlation coefficient value (r) and chi-square value. Steady-state viscosity (η, m.Pa s) was determined from the constant shear section at 100 s^{-1} [31].

2.7. Morphology of developed TH-NPs gel formulations

The morphology of the gel GC-TH-NP-T- was assessed using scanning electron microscopy (SEM JSM-7001 F, JEOL, Tokyo, Japan). For this purpose, the gel sample was dehydrated by incubation at 32 °C for 7 days, then mounted on a conductive bio-adhesive disc (Carbon tabs, Agar Scientific). After this procedure, the sample was carbon-coated (Emitech 950).

Additionally, the gels (GC-TH-NP-T-, GH-TH-NP-T- and GP-TH-NP-T-) were recorded with optical microscopy X63 (LEICA DFC300FX, Leica Microsystems, Wetzlar, Germany), to compare the gel matrix of each developed semi-solid formulation and the system homogeneity [32].

2.8. Ex vivo skin permeation studies

Studies of the skin permeability of TH-NPs gel formulations were carried out using ex vivo human skin explant obtained from plastic surgery of the abdominal region of a healthy female, with the consent of the donor, under an experimental protocol approved by the Bioethics Committee of the Barcelona-SCIAS Hospital (Barcelona, Spain). The following procedures were carried out using Franz Cells (FDC-400, Vidra-Foc, Barcelona, Spain) with vertical diffusion, placing the skin samples between the donor/receptor compartment with the SC facing up. The skin samples used were either 0.4 mm thick (epidermis + dermis) or 1 mm thick skin (including the fat tissue). Selected semi-solid formulations containing TH-NPs were applied onto the skin and allowed permeation for 24 h at 32 °C, maintaining sink conditions.

For the qualitatively skin penetration, the ex vivo skin explant was obtained from the hospital and immediately, the fat tissue was removed manually with sterile surgical razors. Fresh skin samples were cut and directly placed on the Franz Cell using PBS as the receptor medium. Thus, skin samples were washed and cut into small pieces, fixed for 2 h with paraformaldehyde and glutaraldehyde (4 % and 2.5 %, respectively, prepared in sodium cacodylate buffer (0.1 M) pH 7.4, postfixed with osmium tetroxide (1 %) for 2 h at 4 °C and stained in uranyl acetate (0.5 %) for 45 min at 4 °C. Dehydration was performed by increasing alcohol gradients, then samples were dried at critical point (Emitech K850), mounted on a conductor adhesive disc (Carbon tabs, Agar Scientific), and coated (carbon) under evaporation (Emitech 950). Images were visualized by SEM (Jeol JSM-7001F) [33].

For the quantitative analysis of TH-NP skin penetration from the gel formulations, the experiments were assayed following a similar methodology previously described by our group [17]. The receptor medium was filled with Milli-Q water:Transcutol P® (50:50) and the aliquots (300 μL) were collected at selected times for 24 h, being replaced with the same medium. These were analysed by HPLC (High performance liquid chromatography, Waters 2695) adapted to UV detector, using acetonitrile:water (mobile phase) under gradient conditions during 20 min, and a Kromasil® column (C18, 5 μm, 150 × 4.6 mm). TH was detected at wavelength of 275 nm. The skin permeation parameters were calculated with the Eq. (1):

$$J = Kp.C_o \quad (1)$$

where J is the flux, Kp is the permeability coefficient and C_o is the initial concentration of TH.

For TH quantification from the total amount retained inside the skin, TH was extracted using 2 mL of ethanol:water (50:50) under sonication (20 min) in an ultrasonic bath (JP, Selecta) and measured using HPLC. Prior to that, skin samples were washed with sodium lauryl sulphate (0.02 %), rinsed with distilled water, dried, cut, and weighted. Statistical analysis for the experiments were performed either by one-way ANOVA Tukey's multiple comparison test or unpaired t test.

2.9. In vitro release studies

The release of TH-NPs incorporated into gel formulations were executed in Franz cells using a methylcellulose membrane (Dialysis Tubing – ViskingCode DTV12000.03.000, Size 3, Inf Day 20/32″– 15.9 mm, MWCO– 12–14.000 Da, Liverpool Road, London, UK) placed between donor/receptor compartments (2.54 cm^2). 300 μL aliquots were collected and replaced with receptor medium. The method used was previously described elsewhere [17]. Data were analysed by HPLC and adjusted to variable mathematical models using GraphPad® Prism 6 [32]. Statistics analysis were performed using GraphPad® Prism 6, one-way ANOVA Tukey's multiple comparison test or unpaired t test.

2.10. In vitro antimicrobial efficacy

A quantitative analysis of the antimicrobial activity of TH and TH-NPs gel formulations, was determined by the time-kill curve of the products in contact with the microorganisms [34]. The time required, under certain conditions, to reduce a microbial population by one

logarithm, or 90 % of its initial value, is known as the D-value, or decimal reduction time [35]. This study was assayed following the methodology described in our previous work [17]. Briefly, gel formulations containing TH 0.5 % were diluted in milli-Q sterile water to 0.1 % (1000 μg/mL) representing twice and 4x the MIC values, for *S. epidermidis* and *C. acnes*, respectively. Previously, *S. epidermidis* was grown overnight at 37 °C, cultured in MHB medium and *C. acnes* was cultured in BHI medium for 48 h at 37 °C under anaerobic conditions using parches (AnaeroGen®, Oxoid, Basingstoke, UK) and indicator (Oxoid, Basingstoke, UK). Inoculum of each bacteria strain was prepared in PBS at 10^8 CFU/mL and used to inoculate (100 μL) each experimental sample of 10 mL, incubated at 32 °C. At determined times, 1 mL of each sample was neutralized in 9 mL of Berens diluent for 15 min, followed by subsequent 10-fold PBS dilutions. Drop count method (10 μL) was carried out in CRM plates for *C. acnes* and TSA agar plates for *S. epidermidis*, incubated at 37 °C as described above. Results were determined as bacteria viability along time (logCFU/h). The decimal reduction time (D) was calculated using the Eq. (2):

$$D = 1/b \quad (2)$$

where *D* is the time taken to reduce the microbial concentration (CFU/mL) by one logarithm of its initial value and *b* is the slope of the time-kill curve. Statistics analysis were performed using GraphPad® Prism 6, t student unpaired test.

Additionally, a suspension test was performed with selected gels, where the quantitative and qualitative analysis of the antimicrobial activity were carried out by microbial count and SEM as previously described [17]. Briefly, *C. acnes* was cultured in BHI using a shaker incubator (Innova® 4080, New Brunswick Scientific). Then, 100 μg of gels or sterile distilled water (control), were added to 900 μL of concentrated inoculum, followed by incubation for 1 h at 37 °C under controlled shaking. From this point, samples for quantitative analysis were neutralized in Berens diluent for 15 min, then transferred to CRM agar plates and incubated at 37 °C under anaerobiosis for microbial count. For the samples prepared for SEM, centrifugation (10,000 xg for 5 min) was carried out (Centrifuge 5415 C, Geratebau Eppendorf, GmbH, Germany) and supernatants were discarded. The concentrated pellets were transferred into poly-l-lysine coated coverslips and kept at room temperature for 24 h [36]. Samples were fixed for 4 h with 4 % paraformaldehyde and 2.5 % glutaraldehyde (phosphate buffer 0.1 M pH 7.4), then post-fixed with 1 % osmium tetroxide (with potassium ferrocyanide) for 1 h, at 4 °C. After dehydration with alcohol gradients, samples were dried at critical point (Emitech K850), mounted on a conductor adhesive disc (Carbon tabs, Agar Scientific), followed by carbon coating under evaporation (Emitech 950). Images were analysed by SEM (Jeol JSM-7001 F).

2.11. In vitro skin-cell viability assay

The cell viability proliferation activity of the pre-gel aqueous formulations containing surface functionalized TH-NPs was studied in human epidermal keratinocyte cell line (HaCaT) by MTT assay. Cells were cultured in a supplemented (10 % fetal bovine serum, 2 mM l-glutamine, 100 units/mL penicillin G and 100 μL/mL streptomycin) DMEM high-glucose and adjusted to 2×10^5 cells/well density using an automated cell counter (Invitrogen® Countess®, ThermoFisher Scientific, Karlsruhe, Germany). Cells were seeded (100 μL) in 96-well plates, and incubated for 24 h (37 °C, 5 % CO_2) with selected samples concentrations (0.0002 – 0.01 % TH). Then, MTT (0.25 %) was added to the well, further incubated for 2 h, followed by replacement of the medium by DMSO (100 μL) [36]. Measurements of viable cells were determined in a Modulus® Microplate Photometer (Turner BioSystems Inc., Sunnyvale, CA, USA), at 570 nm wavelength. Results were plotted as percentage of viable cells relative to untreated ones [18]. Statistics were applied using GraphPad® Prism 6, unpaired t test, comparing both formulations at each concentration tested.

2.12. In vivo skin biomechanical properties (and skin tolerability)

The biomechanical properties of carbomer gels were assessed by trans-epidermal water loss (TEWL) measurement DermaLab® module (Cortex Technology, Hadsund, Denmark) and skin hydration was measured using a Corneometer (CM 825, C+K electronic Gmbh, Germany). The Ethics Committee of University of Barcelona has previously approved this study, corresponding to the code IRB00003099 (30th January of 2019). The measurement was determined in the forearm before (basal levels) and after the gel application for 2 h, under a climate-controlled room (25 ± 2 ºC, relative humidity 45 %) [32]. Prior to the measurements, volunteers (n = 12) were allowed climate-adaptation (30 min, approximately). The panel selected was aged 25 – 45 years old, presenting normal skin conditions.

The skin sebum was quantified in (μg/cm^2) selected volunteers (n = 10) with acne-prone skin and/or oily, aged 20 – 40 years old, using Sebumeter (Cutometer® dual MPA 580, C+K electronic Gmbh, Germany). Measurements were taken on the forehead for average basal level prior and after the gel application for 1.5 h. Statistics were performed using GraphPad® Prism 6, applying statistical ANOVA non-parametric system, Wilcoxon paired test.

3. Results and discussion

3.1. Physicochemical characterization of TH-NPs gel formulations

In order to evaluate the physicochemical characteristics of the surface functionalized developed TH-NPs, the semi-solid formulation gelled with carbomer was selected since it was the most appropriate to undergo dilutions. The Z_{av} (nm) and the PI were measured before and after incorporation into the gel matrix, being values for the aqueous (pre-gel) and semi-solid dosage forms, respectively (Table 1). By comparing the Z_{av} of GC-TH-NPs with values of TH-NPs suspensions, the mean size has slightly increased for negatively charged TH-NPs, while for the positively charged one, the increase of particle size was higher. The increase of particle size by surface functionalization with chitosan in a different nanosystem was also observed by other researchers [37,38]. Moreover, previous authors reported an electrostatic interaction between the chitosan protonated amine (NH3 +) and the carbomer carboxylate (COO–) [39]. Therefore, this might be the reason why these particles, containing CS on the surface, presented higher Z_{av} when incorporated into the gel. In the case of the PI, data presented a considerable increase for all formulations tested, since the overall measurement includes higher particle sizes from the macro gel matrix dispersion. For this

Table 1
Physicochemical characterization of TH-NPs before and after incorporation into carbomer gels.

	Aqueous		Gels	
	Z_{av} (nm)	PI	Z_{av} (nm)	PI
GC-TH-NP-T-	178.0 ± 0.7	0.089 ± 0.012	239.6 ± 56.6	0.367 ± 0.089
GC-TH-NP-L-	215.8 ± 9.7	0.063 ± 0.018	333.3 ± 67.1	0.440 ± 0.081
GC-TH-NP-P-	180.2 ± 6.5	0.066 ± 0.037	232.9 ± 67.2	0.387 ± 0.098
GC-TH-NP-P-C+	316.5 ± 3.8	0.123 ± 0.035	551.0 ± 47.2	0.440 ± 0.067

reason, the formulations selected to carry out the majority of the experiments developed in this work were GC-TH-NP-T- and GC-TH-NP-P-, due to their main similarity on morphometry, compared to the other surface-functionalized TH-NPs developed, as shown in Table 1.

3.2. Stability of TH-NPs gel formulations

In order to study the stability of the developed formulations, different physicochemical characterization techniques were used. To predict the physicochemical stability of TH-NPs as aqueous and semi-solid dosage forms (GC, GH and GP), TH-NP-T- was chosen, since the most adequate long-term stability previously obtained by Turbiscan Lab® Expert corresponded to this formulation [17]. Thus, the measure of the backscattering profile was acquired to evaluate the short-term stability during storage (Fig. 1). It can be observed that, for the pre-gel aqueous formulation, the NPs may lead to sedimentation along storage (Fig. 1A). However, it was previously reported in our earlier work that this phenomenon is reversible by agitation, without affecting its morphometry [17]. Moreover, it can be observed that when TH-NPs were incorporated into the hydrogels (GC, GH or GP), the BS signal was stabilized by the macrogel matrix surrounding the dispersed TH-NPs (Fig. 1B–D, respectively).

In order to predict a long-term physical stability of the surface modified TH-NPs, the study was performed under accelerated conditions by storing the hydrogels at variable temperatures. For this, carbomer gels, adjusted to pH 6–6.5, were used to incorporate the developed PLGA NPs, since GC is pH dependent, and hence, to evaluate any possible destabilization during storage. The appearance of the gels stored at 4 °C and at RT (23 ± 2 °C) indicates a semi-solid consistency. In contrast, samples stored under accelerated conditions (at 30 and 40 °C) turned into slightly fluid gels, where the higher the storage temperature, the highest the fluidity. The reason for this loss of consistency may be related to the slight decreased pH value, just below pH 6 (Fig. 1E). This physicochemical modification of the gel matrix might be due to the carbomer gelling properties, which are pH dependent, and therefore, this may cause a viscosity decrease. This may be associated to a partial hydrolysis of PLGA into its monomers, lactic and glycolic acids, which may led to a slight decrease of the pH of the formulation, and therefore, destabilizing the gel matrix. In fact, PLGA NPs have previously demonstrated to suffer a degradation process when stored at high

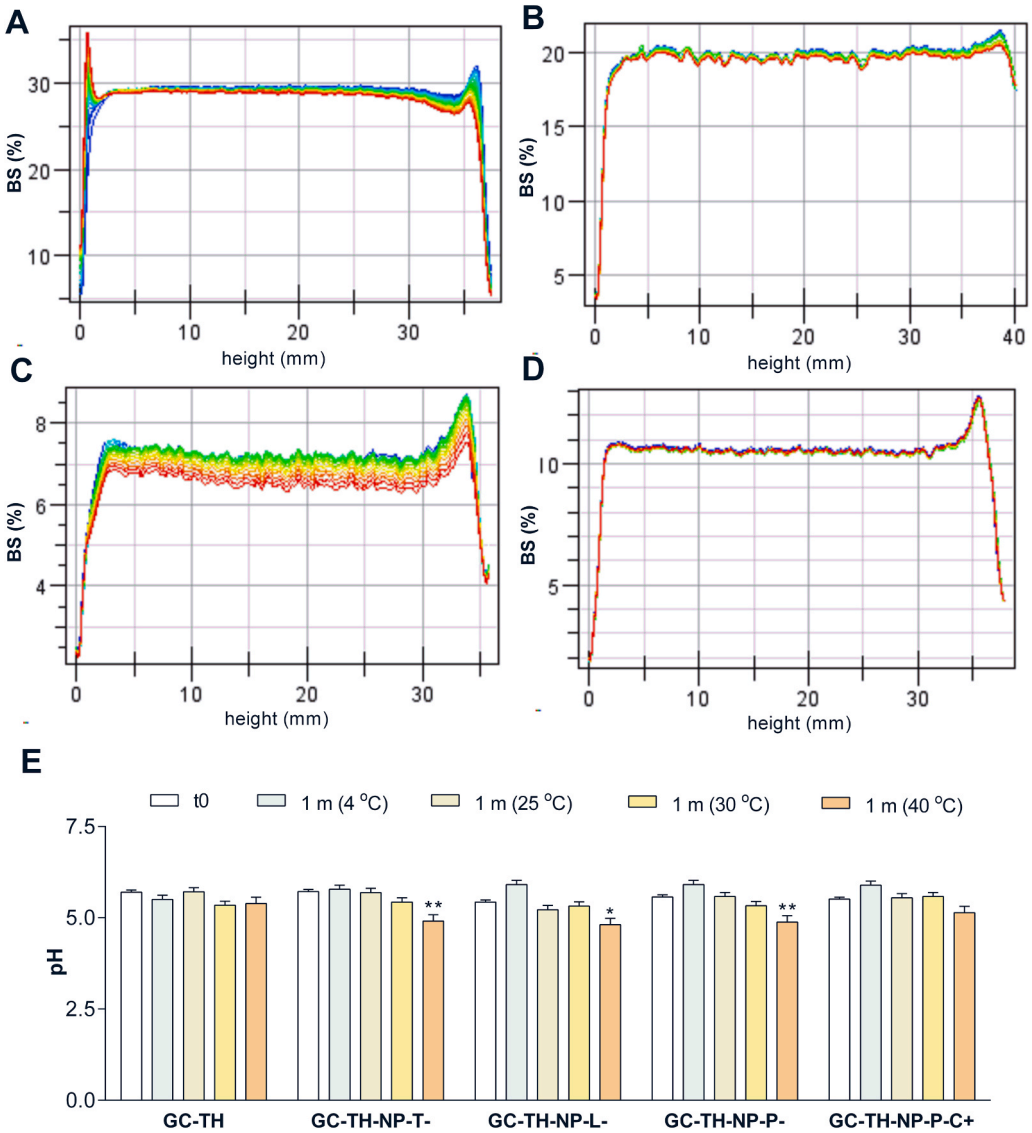

Fig. 1. Backscattering signal of (A) TH-NP-T- (B) GC-TH-NP-T-, (C) GH-TH-NP-T- and (D) GP-TH-NP-T- measured by TurbiscanLab®; scans were performed from the bottom to the top of the vial, measured every hour for 24 h, represented from blue to red lines. (E) Accelerated stability of carbomer gels of variable surface modified TH-NPs after 1 month at different storage conditions, measuring pH. Data expressed as mean ± SD. Statical analysis performed as unpaired t test (*$p < 0.05$).

temperatures [40,41]. On the other hand, other authors previously stated that NaOH is known to catalyze the hydrolysis of PLGA co-polymer by ester cleavage [42]. For this particular case, a suitable alternative would be to increase the pH of carbomer gels using a different neutralizer. In the case of the NPs surface-covered with CS, the pH was diminished but did not present relevant differences under accelerated conditions. In this case, this polysaccharide molecule attached to the NPs surface might have helped to stabilize and/or protect the PLGA against partial hydrolysis, also probably due to the acidic conditions by the use of acetic acid. In fact, the accelerated stability of TH-NP-T- (aqueous suspension, absent of pH neutralizer) was previously studied, where the pH would decrease slightly, but the morphometry and morphology were stable for 3 months at RT and 37 °C, and for 12 months at 4 °C, presented in our previous work [17].

On the other hand, all gels of surface functionalized TH-NPs stored at 4 °C had maintained their semi-solid stability for 12 months, as well as their pH above 6 under storage. This agrees with the stability studies performed in a previous work with the aqueous variable surface modified TH-NPs, which were stable for 6 months at 4 °C [18]. This confirms that PLGA NPs stored at low temperatures maintain the polymer stability and avoiding a possible hydrolysis.

The long-term stability of GC-TH-NP-T- and GC-TH-NP-P- was also studied for 6 months at room temperature and data of the physicochemical parameters evaluated are displayed in Table S1. It can be observed that the TH-NPs morphometry inside the gel matrix has maintained its initial parameters for 6 months.

After that, the gels lost viscosity within 6 months, turning into slightly fluid formulations. This result agrees with the ones under accelerated storage conditions. When GC-TH-NP-T- was stored at 4 °C, it maintained pH and semi-solid appearance for 12 months, confirming that low temperatures increase the shelf-life of PLGA-NPs. On the other hand, formulations of TH-NP-T- incorporated into HPMC or Pluronic gels maintained stability (pH and semi-solid appearance) stored at RT for 12 months. For all the samples evaluated, there were no changes in color or odor of the developed formulations.

Additionally, carbomer gels of TH-NP-T- and TH-NP-P- were formulated adjusting the pH between 5 and 5.5, and followed long-term stability (6 months) evaluating the appearance (consistency/fluidity), pH and organoleptic properties. In this particular case, the formulations at its initial evaluation presented a slightly fluid appearance (serum-like formulation), specifically to observe its behavior according to the pH. The initial pH value was 5.35 and after 6 months it was 5.21. The consistency of the gel was the same as its initial state (slightly fluid gel). Therefore, for this specific type of carbomer formulation (incorporating PLGA-NPs), if the initial state shows fluidity, the rheological properties could be maintained. Also, the pH 5 – 5.5 is the most indicated for acne and oily skin conditions.

The microbial preservative activity stability was studied for the gels selected for the efficacy studies (GC-TH, GP-TH, GC-TH-NP-T-, GP-TH-NP-T-, GC-TH-NP-P- and GP-TH-NP-P-). These presented preservative activity after 6 and 12 months, stored at RT and 4 °C, respectively, evaluated for bacteria, yeast, and fungi growth. Despite it is worth to

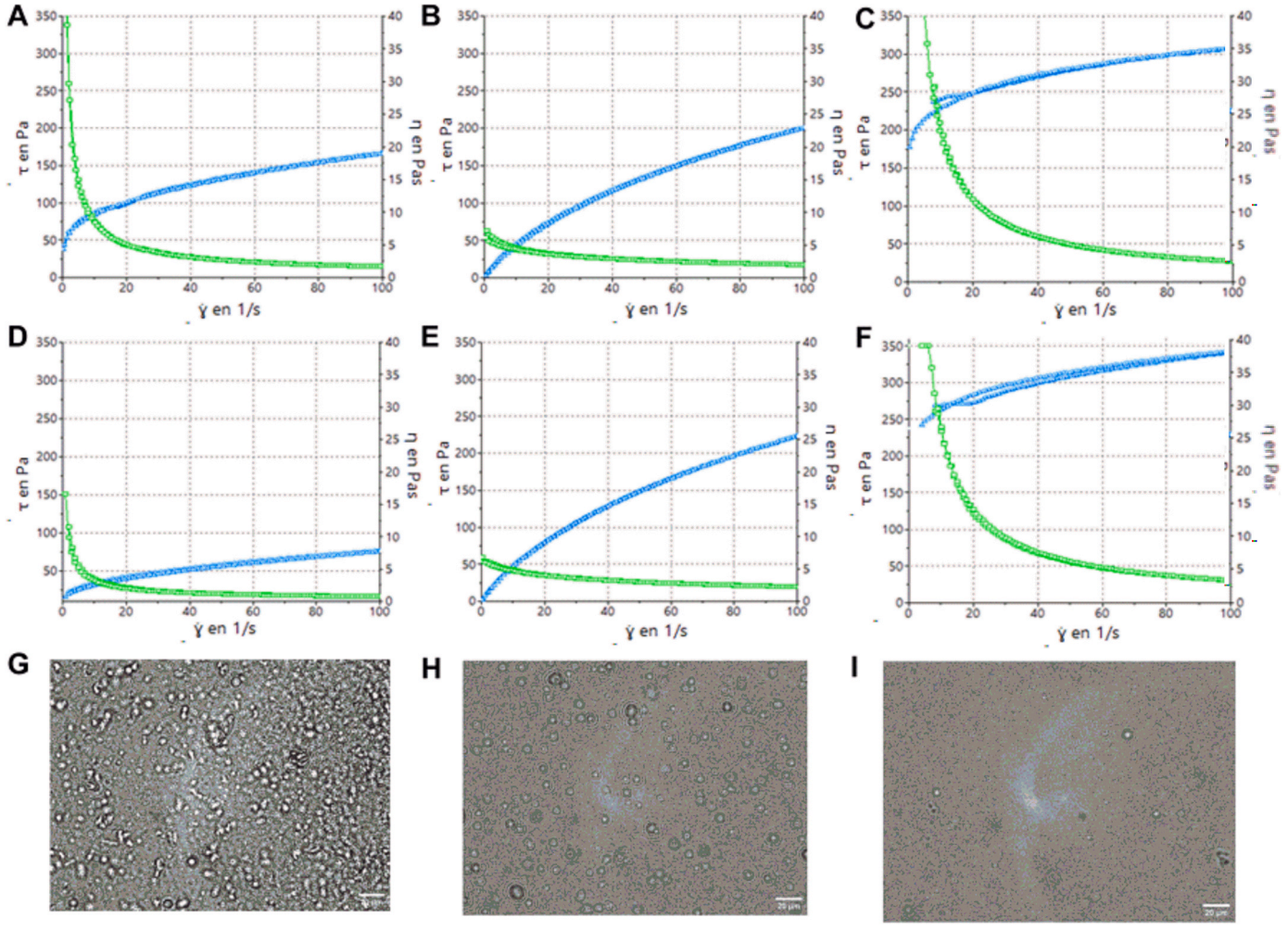

Fig. 2. Rheological behavior of A) GC-TH, (B) GH-TH, (C) GP-TH, (D) GC-TH-NP-T-, (E) GH-TH-NP-T, and (F) GP-TH-NP-T-. Data was fit to cross mathematical model corresponding to a pseudoplastic flux. (G-I) Optical microscopy micrographs (x63) for GC-TH-NP-T, GH-TH-NP-T- and GP-TH-NP-T-, respectively.

mention that no challenge test was performed to test their preservative capacity upon induced microorganisms, TH and TH-NPs formulated with polymeric gelling agents, presented good microbial preservative activity during storage. The use of natural antimicrobial agents in cosmetic formulations, reduced the need of strong chemical preservative systems, which makes it favourable to be classified as natural products. Other authors also reported previously the preservative activity of thymol in cosmetic formulations [43].

3.3. Rheological studies of TH-NPs gel formulations

In order to study rheological behavior of TH-NPs semi-solid formulations, samples were dispersed into variable gels to observe their behavior at different dosage forms. The rheological profile of GC-TH, GH-TH, GP-TH, GC-TH-NP-T-, GH-TH-NP-T- and GP-TH-NP-T- are illustrated in Fig. 2A–F, respectively, and the corresponding parameters are shown in Table S2**.** The results of the steady-state rheological measurements showed that the formulations were dependent on shear rate. All formulations exhibited non-Newtonian rheological behaviour, all showing a consistent decrease in viscosity with increasing shear rate from 1 to 100 s^{-1}, The rheological flow was found to be pseudoplastic shear thinning for GCs and GHs, and plastic for GPs. Mathematical models that best fit experimental data were the Cross Eq. (3) that provides a general model for pseudoplastic materials and Herschel-Bulkley Eq. (4) for plastic profiles.

$$\eta = \eta\infty + \frac{\eta o - \eta\infty}{(\tau\gamma)^m} \tag{3}$$

$$\eta = \frac{\tau 0}{y + K^* y^{(m-1)}} \tag{4}$$

being η the apparent viscosity, η^0 and $\eta\infty$ asymptotic viscosity values at very low and high shear rate, respectively, τ the shear stress (Pa), γ the shear rate (1/s), m the flow index, $\tau 0$ the shear stress (Pa) when deformation velocity tends to zero, y is the deformation velocity and K consistency coefficient.

Concerning viscosity measurements, GP-TH-NP-T- and GP-TH displayed a maximum viscosity value of 3,43 ± 0,01 and 3,08 ± 0,00 Pa.s, respectively, and GC-TH-NP-T- was the formulation who showed a lowest viscosity (0,77 ± 0,00 Pa.s). The last can be explained by the fact that all formulations were adjusted to pH 5 – 5.5, and in this condition, a slightly fluid consistency was found for GC containing NPs. Thus, it can be observed that GC-TH showed higher value of viscosity (2-fold) compared to the one with the NPs incoporated. This could be attributed to the lower pH value present in the pre-gel, since PLGA-NPs present low pH values (due to its monomers composition, lactic and glycolic acids). In contrast, comparing GH or GP incoporating TH or TH-NPs, the viscosity values are more similar, since those gelling agents are not pH dependant, in fact, being the ones containing the NPs the ones with slightly higher viscosity. Nevertheless, it is clear to observe in Table 3 that the viscosity increase as the increase of the concentrations of the gelling agents used (GC<GH<GP).

3.4. Morphology of TH-NPs gel

All gel formulations were observed by optical microscopy (X63) and micrographs are shown in Fig. 2G–F, for GC-TH-NP-T-, GH-TH-NP-T- and GP-TH-NP-T-, respectively. It can be observed that all formulations displayed different matrix structures. In the case of GH and GP, they present a thin and flat homogeneous framework with spaced droplets within the matrix, which could be associated to their higher viscosity profile. In the case of GC, the framework is homogeneous but showing an uneven surface, and presenting more droplets brought together with higher intensity. The lower the viscosity, the higher the number of droplets, which could also be related to the spaces between the matrix, that allows higher flexibility and mobility of the polymeric frame. Furthermore, the nanostructure morphology of selected gels was also visualized by SEM, illustrating the cross-linking structure of GC-TH (Fig. 3A–B) and the NPs within the matrix, for GC-TH-NP-P- (Fig. 3C–D) and GC-TH-NP-T- (Fig. 3E–F). It can be observed that the GCs matrix structures form three-dimensional nanometric cross-linked filaments with micronized pores, and the NPs are found embedded in the threads.

3.5. Ex vivo skin permeation of TH-NPs gel formulations

The skin penetration of TH and TH-NP-T- were evaluated in ex vivo human skin using different types of dosage forms (aqueous and semi-solids). For the qualitative analysis, GC-TH-NP-T- was evaluated after 24 h of contact with the skin and observed by SEM. Images of untreated and treated skins are displayed in Fig. 3G–H, respectively. As observed, the gel matrix containing TH-NPs was found within the skin tissue after

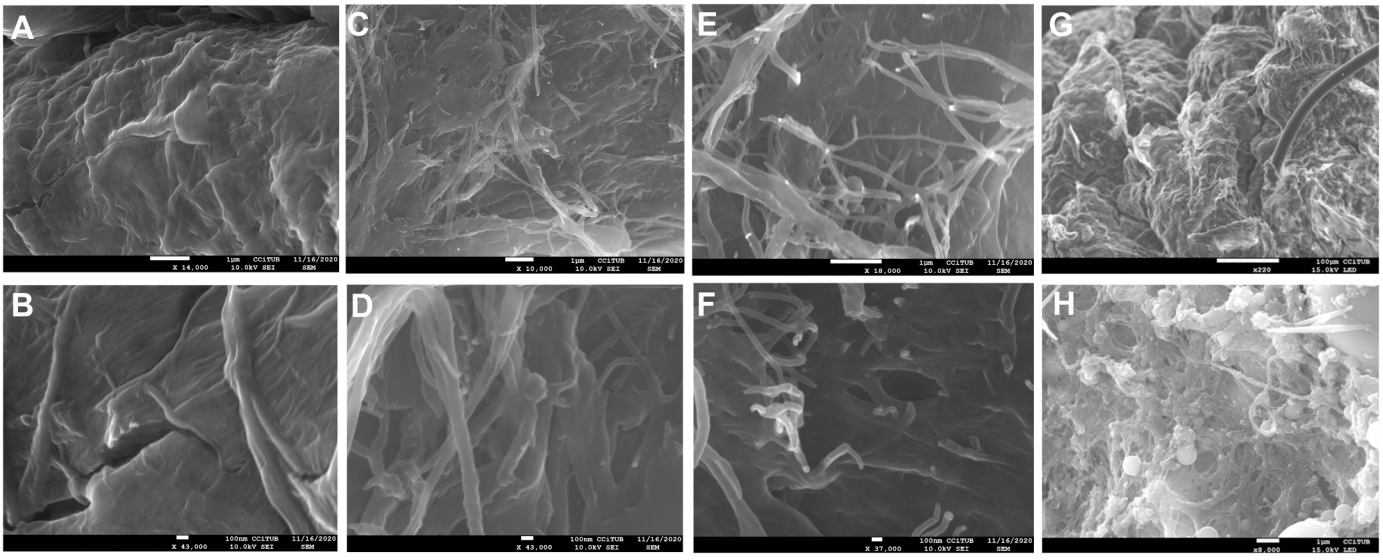

Fig. 3. Scanning electron microscopy image of (A/B) GC-TH (—1 μm, x 14.000 / 100 nm, x 43.000), (C/D) GC-TH-NP-P- (—1 μm, x 10.000 / 100 nm, x 43.000),) and (E/F) GC-TH NP-T- (—1 μm, x 18.000 / 100 nm, x 37.000). SEM images of ex vivo skin permeation for 24 h for (G) untreated (—100 μm, X220) and (H) GC-TH-NP-T- (—1 μm, x10.000). respectively.

24 h of contact. The degradation of a carbomer gel was previously found to be within 24 h [44]. The lower viscosity of the gel could enable its penetration and the bioadhesive properties would maintain the gel within the skin tissue [45].

For the quantitative analysis, the first study evaluated the variability of TH-NP-T- permeating through the skin, when incorporated into 3 different semi-solid excipients (GC, GH and GP), and the variable parameters obtained are shown in Table 2. The highest values (*J*, *Kp* and *Qp*) were found for GC-TH-NP-T-, being very similar to GH-TH-NP-T-. Meanwhile, GP-TH-NP-T- presented the lowest values, statistically significant ($p < 0.05$), where the permeation rate can be observed to be very slow, compared to the other ones. In the case of the total amount retained inside the skin (*Qs*), the highest value obtained was also for GC-TH-NP-T-, presenting statistically significant differences ($p < 0.0001$) (Fig. 4A). Therefore, the excipient of the formulation is a relevant tool for the desirable type of penetration rate, depending on the treatment. In fact, the viscosity of the semi-solid formulation is also a parameter that affects the penetration rate. In the case of GP-TH-NP-T-, previous authors also found very low concentrations in the receptor fluid and retained inside the skin, after 24 h penetration for pluronic gel, while for the free-aqueous dosage form, the values were much higher [46]. These findings agree with the release profile of GP gels obtained in the next Section (3.6).

The second study, based on the results obtained for the study above, consisted of comparing TH and TH-NP-T- as aqueous and carbomer gel formulations (Table 2). These formulations were selected due to the results obtained above and since they present lower viscosity, which would facilitate the skin permeation and retention along the experiment conditions. The sustained penetration rate of encapsulated TH was clearly observed for both dosage forms. Statistically significant differences were obtained for the flux (*J*), permeation constant (*Kp*) and total amount penetrated in 24 h (*Qp*), between aqueous formulations ($p < 0.0001$, $p < 0.0001$, and $p < 0.05$, respectively). In the case of their carbomer gel formulations, the permeation parameters obtained also presented statistically significant differences between them ($p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively). Additionally, it can be also observed that both gels presented significant differences ($p < 0.0001$), compared to their aqueous forms performing a much slower permeation rate. The cumulative amount found in the receptor fluid was also higher for the aqueous formulations than the gels, being significantly higher for TH compared to TH-NPs in both cases (aqueous and semi-solid) ($p < 0.05$). Moreover, the amounts retained inside the skin were found to be higher for the encapsulated formulations than for free-TH, presenting statistically significant differences between them ($p < 0.01$) (Fig. 4B).

In the case of the study performed with the full skin, including the fat-tissue, the cumulative amount found in the receptor fluid for GC-TH and GC-TH-NP-T- were similar, being 35.56 ± 3.67 and 35.47 ± 1.55 μg/cm^2, respectively. However, the total amount retained inside the skin (Fig. 4C) was found to be statistically significant higher for TH ($p < 0.01$), which raises the idea that GC-TH could be also found retained in the fat tissue. Meanwhile, GC-TH-NP-T- could be possibly retained only along the epidermis and dermis layers, resulting in lower total amount found inside the total skin sample. It has been previously stated that the fat tissue may serve as a deep compartment for the drug, delaying entry into the blood [48]. Therefore, this would increase the time retained inside the skin, before (or without) reaching the fat tissue, remaining where the targeted treatment requires therapeutical activity. For this reason, the encapsulation of TH has shown improved performance for dermal applications, which would be suitable for its therapeutic efficacy. Additionally, in our previous work, the skin permeation of TH-NPs surface functionalized (listed before) was evaluated by confocal microscopy, where the route of penetration of all those were found to be through the hair follicle [18].

3.6. In vitro release of TH-NPs gels

The in vitro release was investigated for TH and TH-NP-T- aqueous form, carbomer gels and pluronic gels, for 72 h. The release of TH-NP-T- showed a sustained release rate compared to TH, both adjusted to the hyperbola Eq. (5) (Fig. 4D). As it can be observed, the release kinetic of free TH was very fast, where the steady-state was reached just after 12 h, meanwhile, the encapsulated TH-NP-T- provided a continuous release profile during the entire length of the study, which would be only reaching the stead-state after 72 h. Comparing the total amount released at 24 h ($Q_{r\_24\,h}$), it presented statistically significant differences between them ($p < 0.01$) and for the dissociation constant (*K*) ($p < 0.0001$) which is the time taken to achieve ½ of the maximum release value (Q_{max}) (Table S3). Similar results could be observed for both formulations when incorporated into the carbomer gels, where GC-TH-NP-T- was more sustained than GC-TH (Fig. 4E). Comparing the kinetics of the gels, GC-TH release was extremely fast, whereas GC-TH-NP-T- was even more sustained compared to TH-NP-T- (aqueous NPs). The $Q_{r\_24\,h}$ for the gels presented statistically significant differences between them ($p < 0.0001$) as well as for *K* ($p < 0.01$). Moreover, GC-TH-NP-T- release kinetic was significantly slower compared to TH-NP-T-, with (*K*, $p < 0.0001$) and total amount release after 24 h ($p < 0.01$), meanwhile, GC-TH and TH rate release and total amount were not significantly compared (Table S3). Overall, when comparing the values ($Q_{r\_24\,h}$), between the formulation types (aqueous or gels), GC-TH-NP-T- presented 2X slower amount of TH-NP-T-release, providing an even more sustained/controlled kinetics, meanwhile TH and GC-TH were similar and found already on the plateau. These findings have also been demonstrated by other authors due to the fact that TH is first released from the NPs, and then contacts with the gel, that also causes a delay of

Table 2
Ex vivo skin penetration parameters of different gels containing TH-NP-T- and different TH and TH-NP-T- dosage forms.

Gels containing TH-NPs				
Parameter	GC-TH-NP-T-		GH-TH-NP-T-	GP-TH-NP-T-
J (μg/cm²/h)	1.58 ± 0.21		1.34 ± 0.14	0.28 ± 0.06 *a b*
Kp (cm²/h)	6.22E-04 ± 8.80 E-05		5.38E-04 ± 5.60E-05	1.11E-04 ± 2.43E-05 *a b*
Qp (μg/cm²)	46.15 ± 6.82		41.09 ± 4.89	33.52 ± 3.82 *a*
SSD (p < 0.01)	*a*		*b*	*c*
Dosage forms TH				
Parameter	**TH**	**TH-NP-T-**	**GC-TH**	**GC-TH-NP-T-**
J (μg/cm²/h)	9.36 ± 0.31 *e f g*	2.95 ± 0.15	2.53 ± 0.77	1.37 ± 0.20 *e f*
Kp (cm²/h)	2.74E-03 ± 1.240E-04 *e f g*	1.18E-03 ± 6.17E-05	1.01E-03 ± 3.08E-04	5.46E-04 ± 8.08E-05 *e f*
Qp (μg/cm²)	85.45 ± 9.63 *e f g*	71.56 ± 10.50 *f g*	38.79 ± 2.98	22.83 ± 3.78 *f*
SSD (p < 0.01)	*d*	*e*	*f*	*g*

J: flux, Kp: permeability constant, Qp: cumulative amount permeated. Statistical analysis one-way ANOVA Tukey's multiple comparison test.

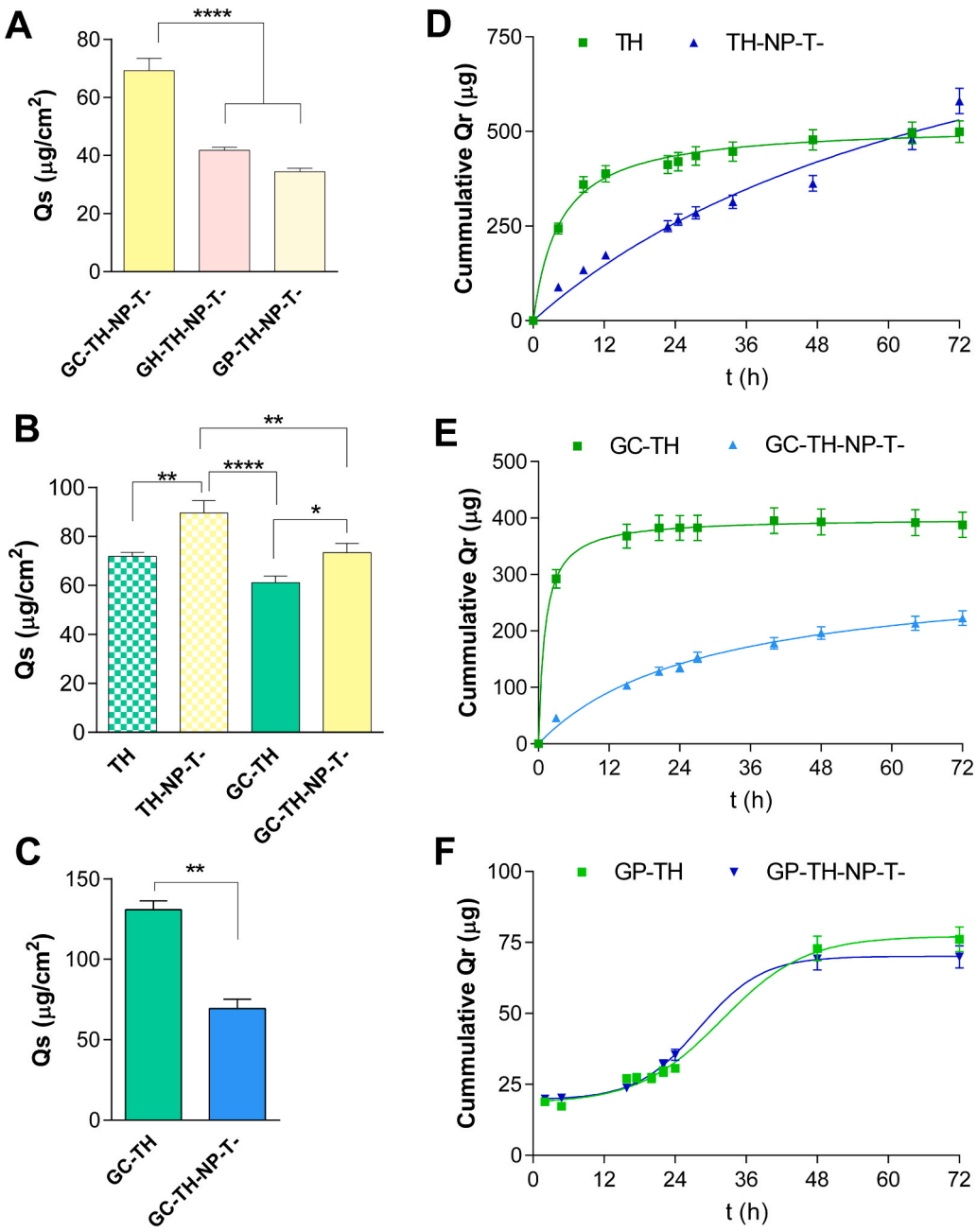

Fig. 4. Ex vivo skin penetration for 24 h with the total amount retained inside the skin (*Qs*) via extraction technique. (A) TH-NP-T- carbomer (GC), HPMC (GH) and pluronic (GP) gels, (B) TH and TH-NP-T- aqueous and carbomer gels, and (C) GC-TH and GC-TH-NP-T- (using full-fat skin tissue). Data are expressed as mean ± SD (n = 3). Statistical analysis one-way ANOVA, Tukey's multiple comparison test, or unpaired t test (*p < 0.05, **p < 0.01 and ****p < 0.0001). In vitro release profile (cumulative amount released (*Qr*) vs time of (D) TH and TH-NP-T- and (E) GC-TH and GC-TH-NP-T-, obtained adjusted to hyperbola equation. (F) GP-TH and GP-TH-NP-T-, obtained adjusted to sigmoidal equation.

release due to the higher viscous matrix [49]. On the other hand, the release of GP-TH and GP-TH-NP-T- (Fig. 4F), adjusted to sigmoidal mathematical Eq. (6), both provided a very slow-rate release with very low cumulative amount released within 24 h, presenting no significant differences between them (Table S4). Pluronic is a thermo-reversive gel, therefore, the viscosity and the gel matrix properties are different at RT than at body temperature. In this experiment (32 °C), the gel matrix would increase viscosity, which might be the influence of the very sustained release of TH. The highest viscosity found for the developed gels was found for GPs, which would agree with these results obtained. Russo et al. also found similar results in drug release comparing aqueous and pluronic gel formulations (at 17 % and 20 % pluronic), where the maximum amount released in 24 h was no more 5 % of the initial dose applied [47]. The release of GHs was not studied since they presented similar permeability profile than GC. In fact, GCs were selected due to higher skin retention of TH. Therefore, it was worthy to evaluate the differences between GCs and GPs, as they present relevant variability on physicochemical, rheological and permeation parameter.

$$Q_r = \frac{Q_{max} \cdot t}{K + t} \tag{5}$$

$$Q_r = Q_0 + (Q_{max} - Q_0) \Big/ (1 + \exp(\frac{K - t}{b}) \tag{6}$$

Where Q_r is the amount of TH released, t is time, Q_{max} is the maximum

amount released, K is the time taken to reach half of the maximum amount of TH released, Q_0 is the lowest amount released and b is the curve of deformation-steepness (slope).

Additionally, comparing results found for the release kinetic of TH from variable formulation types (free/encapsulated, aqueous/gel) with the ones found for the skin permeation studies, it can be associated that the rate of penetration of TH into the skin is directly proportional to the release kinetics of the excipient. In the case comparing the aqueous formulations, the release and permeation kinetics were very fast, whereas for TH-NP-T- it was highly sustained. In the case of the gels, the same feature can be observed, as the carbomer gels presented a decrease in the release and permeation kinetic rates. Moreover, evaluating the pluronic gels behaviour, they present a much slower kinetic, compared to carbomer gels, where minimal amounts were found permeated or released. Therefore, the formulations developed can be applied for a variety of administration needs, either fast to very slow permeation and release rate, where it can be suggested that TH penetration would be determined primarily by its diffusion from the NPs and then from the gel matrix [50]. In this case, for skin acne treatment, a slow-rate release and permeation would be suitable in order to deliver smaller amounts of TH for prolonged timepoints. In this sense, the treatment would be much more effective than that with free TH. Therefore, encapsulation of TH constitutes a feasible strategy for a long-term treatment with increased efficiency.

3.7. In vitro antimicrobial efficacy

The antimicrobial activity of carbomer and pluronic gels of TH and TH-NP-T- were tested in contact with *C. acnes* for 3 h, at concentrations of 1 mg/mL. Results showed that carbomer gels (Fig. 5A) detained the microbial viability of *C. acnes* within 2 h. Meanwhile, pluronic gels (Fig. 5B) performed a sustained behaviour, since this gel formulation at 32 °C increases its initial gellification viscosity. On the other hand, when carbomer gels where tested with *S. epidermidis* (Fig. 5C), GC-TH abolished microbial viability within 3 h, whereas GC-TH-NP-T- still maintained a few colonies alived within 48 h contact. Therefore, nanostructured systems of thymol provide fast activity against the major acne pathogen, while not affecting entirely the healthy skin microbiota.

The results obtained in this study are in agreement with the ones obtained in a previous work with the aqueous forms of TH and TH-NP-T- [17].

However, the carbomer gel formulations provided a slightly faster activity compared to aqueous. Other authors also found that PLGA-NPs presented higher antimicrobial activity when incorporated into a carbomer gel [49].

In contrast, GP-TH and GP-TH-NP presented a sustained antimicrobial activity against *C. acnes*. This is in accordance with the demonstrated slow-rate release from both GPs, and it could be attributed to the viscosity and physicochemical properties of the formulation, since it increases thickness and adhesive when in contact to the skin. Therefore, it would delay the release of TH from the NPs towards the bacterial membrane, providing a delayed activity against *C. acnes*. Further studies to elucidate the differences between GP-TH and GP-TH-NP for prolonged exposure period with the microorganisms would be able to confirm this hypothesis. Since *S. epidermidis* is a much more resistant microorganism and presents a long time-kill curve, this study was not performed with GPs. Previous authors also stated that the higher the polymer gelling agent concentration, provide higher influences on the gel viscosity and bio-adhesiveness, promoting a slower drug release [49]. Therefore, if a very sustained effect is required in vivo, this gel vehicle would be the most appropriate, specially combined with wound healing processes [47].

The antimicrobial activity was also assessed qualitatively by SEM. The structure of untreated *C. acnes* is shown in Fig. 5D, presenting a rod-shape and smooth membrane. When the bacteria were treated with GC-TH (Fig. 5E), the cell envelope was found thick, elongated, and deteriorated, presenting also small blebs on the surface. In the case of treatment with GC-TH-NP-P- (Fig. 5F), the exterior cell membrane has remained smooth, however, the cell envelope was elongated and the surface slightly damaged. These results were similar to the ones obtained with TH and TH-NPs as aqueous forms in our previous work [17]. Additionally, Fig. 5G illustrates the gel matrix, containing the NPs, entrapping bacteria. In agreement with these findings, the effect of GC-TH-NP-P- was slightly less aggressive than GC-TH, where the effect can be also expected to maintain the healthy skin microbiota balanced, by not damaging and abolishing completely the microorganisms, due to their slow-rate release profile.

The quantitative analysis of the suspension test, performed with GC-TH, GC-TH-NP-T- and GC-TH-NP-P- in contact with *C. acnes* for 1 h is shown in Fig. S1A. Results showed statistically significant differences against the control ($p < 0.0001$) for all formulations tested. Although, there was no significance between them when treated for a short period, probably due to the need of TH release from the GC matrix and from the PLGA-NPs. Therefore, the products present sustained, antimicrobial activity without completely abolishing the microorganisms.

3.8. In vitro skin-cell viability assay

The cell viability of selected formulations (diluted pre-gels) were studied in HaCaT cells to evaluate the cytotoxicity for skin applications. Results are showed in Fig. S1B, the lowest value was found only for TH-NP-T-, presenting statistically significant differences ($p < 0.01$) comparing both formulations at the highest concentration tested (0.1 %). Moreover, the viable count was maintained above 70 % for all concentartions tested (thus indicanting that no cytoxicity was caused accoding to ISO 10993-5) [51]. Based on these results, the formulation selected to apply for the in vivo study of biomechanical properties of the gel was GC-TH-NP-P-.

3.9. In vivo skin biomechanical properties (and skin tolerability)

The skin integrity was evaluated for application of GC-TH and GC-TH-NP-P- for 2 h, recording measurements on the forearm of volunteers ($n = 12$) and results are expressed in Fig. 6. Observing the overall results for the hydration of the SC for both products (Fig. 6A–B), it presented minimal variation and no significant statistically differences compared to the basal levels were found. In the case of the TEWL (Fig. 6C–D), values have decreased from the basal measure but only presenting statistically significant differences ($p < 0.05$) for GC-TH-NP-P- after 2 h. Therefore, the products tested do not alter the skin properties but may help to reduce TEWL, which can be favored for dermatological treatments. Results obtained in our previous work showed that the skin penetration route of TH-NPs by confocal microscopy was by the hair follicle but particles were also retained in the SC and primary layer of epidermis presenting a delayed entry of the NPs, due to the slow penetration rate [17]. Ideally, this could be associated to TEWL result obtained, together with the humectant agent used for the formulations tested. Following application of the formulations, there was no discernible skin irritation or erythema, suggesting that both formulations were well-tolerated on the skin.

The skin sebum was measured before and after application of carbomer gels during 1.5 h. Results showed that the sebum reduction activity was found to be higher for GC-TH (Fig. 6E) compared to GC-TH-NP-P- (Fig. 6F). Statistically significant differences ($*p < 0.05$) could be observed, compared to basal level (t0), at all measured times for GC-TH, meanwhile, GC-TH-NP-P- was only significant at 60 min. This could be related to the fast and direct activity of TH free, that might drain part of the sebum content during penetration, due to its highly hydrophobic affinity.

On the other hand, similar activity was obtained comparing GP-TH and GP-TH-NP-P- (Fig. 6G–H), presenting statistically significant differences against the basal level ($p < 0.05$). Observing this, the

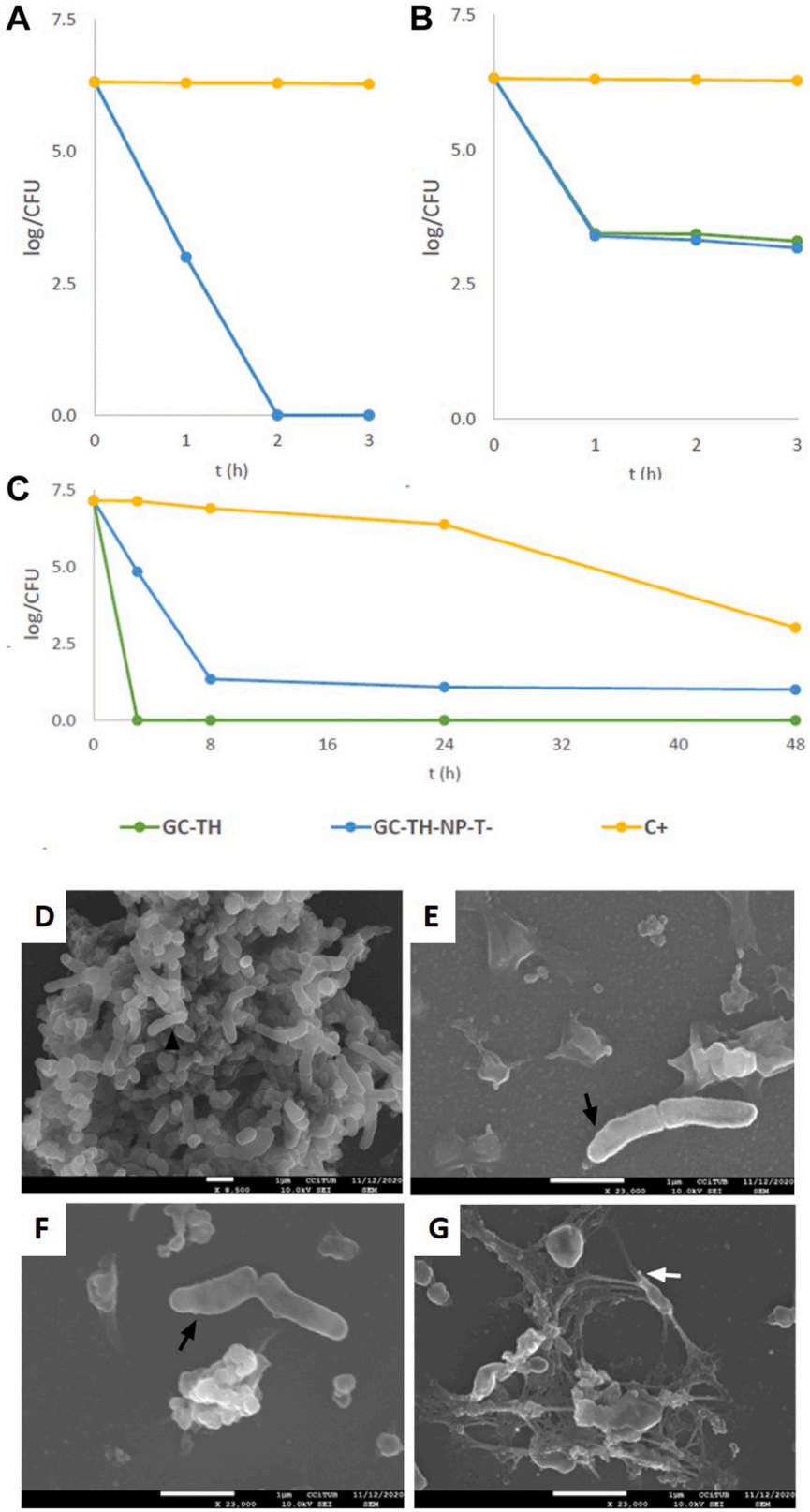

Fig. 5. Antimicrobial assays. A-C) Bacterial viability assay of *C. acnes* for 3 h treated with (A) GC-TH and GC-TH-NP-T- and (B) GP-TH and GP-TH-NP-T-; and of *S. epidermidis* for 48 h treated with (C) GC-TH and GC-TH-NP-T-; D-G) SEM micrographs of *C. acnes* (A) control and treated for 1 h with (B) GC-TH, (C) GC-TH-NP-T- and (D) GC-TH-NP-P-. Black arrows indicate membrane disruption and white arrow points TH-NP-P-.

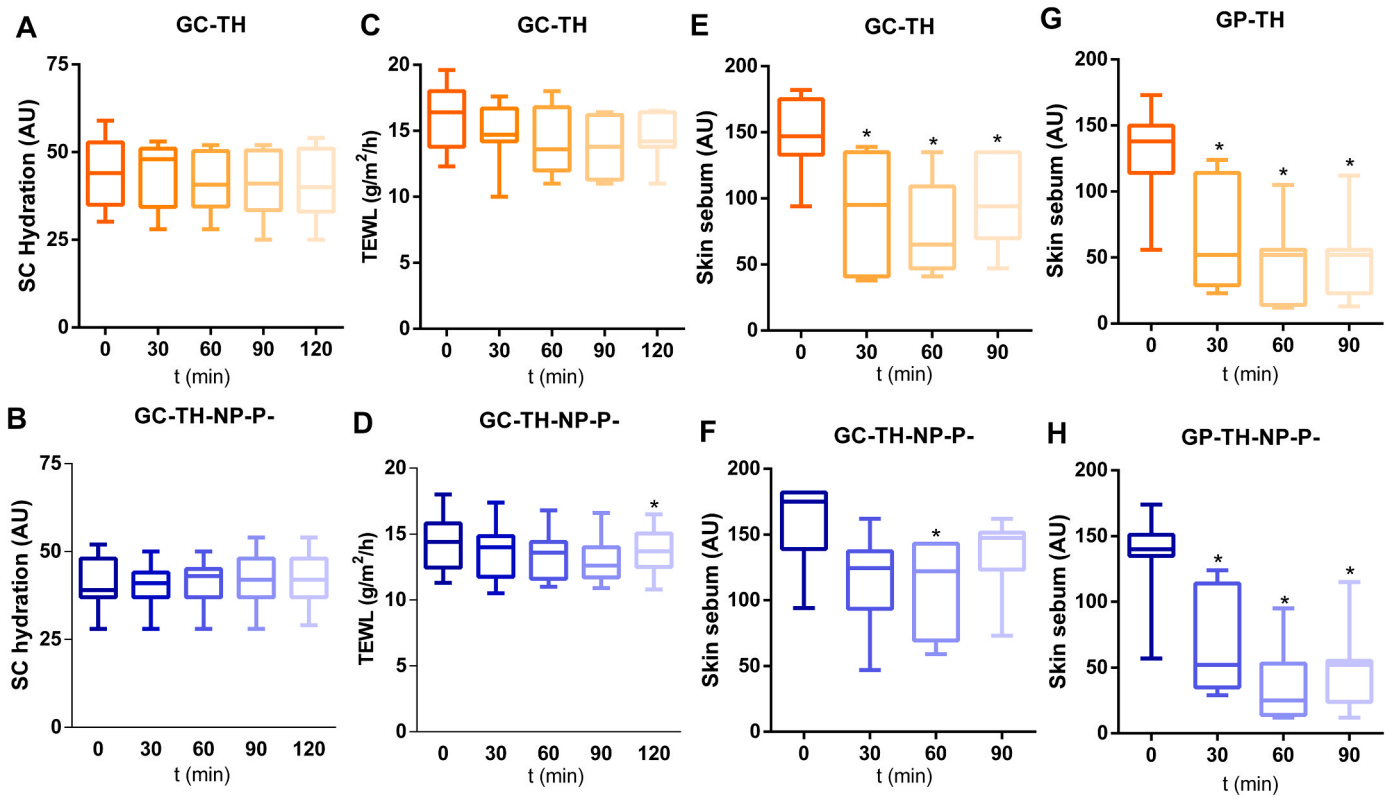

Fig. 6. In vivo biophysical properties of GC-TH and GC-TH-NP-P- measured on the forearm of volunteers (n = 12) for hydration of SC (A-B) and for TEWL (C-D). Statistical analysis performed as mean ± SD values, non-parametric Wilcoxon paired test comparing each measure against basal value (t0). *S*ebum levels measured with Sebumeter® on the forehead of oily skin volunteers (n = 10) for a single application of (E) GC-TH, (F) GC-TH-NP-P-, (G) GP-TH and (H) GP-TH-NP-P-. Statistical analysis performed as mean ± SD values, non-parametric Wilcoxon paired test comparing each measure against basal value (t0).

concentration of pluronic used (20 %) might be the reason for higher sebum-reduction activity, since poloxamers are surfactant molecules which could help to reduce fastly the skin sebum levels. Moreover, higher activity could be observed comparing pluronic gels against carbomer gels. In fact, according to the results obtained for the release and skin penetration of pluronic gels (highly sustained) and the fact that they possess thermo-reversible gelling transition, the gel probably forms a film barrier on top of the skin layer, which may help to regulate the skin loss of water and lipids. Carvajal-Vidal et al. previously stated that pluronic gel on the skin forms superficial thick and dried film adhered to the SC [32]. They found that this effect provided an artifact measured on the skin barrier, where skin moist could not be detected properly, as values of SC hydration were diminished by 40 %. These findings could possibly be attributed to the plastic flow type material that pluronic gels form. For this reason, evaluation of TEWL and skin moisture were not performed in this work. Russo et al. confirmed that pluronic gels hardly penetrate towards damaged skin barrier, which might be related to its enhanced bioadhesive properties and this superficial film formed on the skin barrier [47]. For all formulations, it can be observed that within 90 min, the values started to increase, meaning that the sebum levels are returning to the basal level.

Long-term study with daily applications would be required to find the additional differences between the treatment with one product or the other. According to the results obtained in this current work and previous ones, it seems TH-NPs are great candidates for long-term treatment with potential for better results.

4. Conclusion

In the present study several TH-NPs have been dispersed in three different gelling systems. The formulations showed suitable physicochemical properties and the gels demonstrated the ability to increase the stability of the nanoparticles being the most suitable temperature for storage at 4 °C. Moreover, rheological studies demonstrate an increase in viscosity caused by the gels, which was lower in the case of carbomer. Moreover, the observation of this systems showed 3D cross-linked filaments with the nanoparticles in between. Regarding the therapeutic efficacy, these systems showed promising ability to diminish excessive *C. acnes* but preserve *S. epidermidis*, which would help to regulate the healthy skin microbiota and after the application in human skin, it was confirmed that the systems do not alter skin properties and are able to reduce sebum. As a conclusion, the present TH-NPs dispersed in a semi-solid gel such as carbomer constitute a suitable alternative for acne vulgaris treatment demonstrating suitable antimicrobial and sebum reduction efficacy while preserving skin properties and healthy microbiota.

CRediT authorship contribution statement

Camila Folle has contributed to Investigation, methodology and writing the original draft of the manuscript. Ana M. Marqués has contributed to methodology, investigation and formal analysis of the manuscript. Joaquim Suñer-Carbó has contributed to methodology, formal analysis and review and editing the manuscript. Mireia Mallandrich has contributed to investigation and review and editing of the manuscript. Lyda Halbaut has contributed to formal analysis, investigation and methodology of the manuscript. Elena Sánchez-López has contributed to conceptualization, investigation and writing, review and editing of the manuscript. Ana Laura López-Machado has contributed to investigation and methodology of the manuscript. Josefa Badia has contributed to methodology, investigation and funding of the manuscript. Laura Baldoma has contributed to methodology, investigation

and funding of the manuscript. Marta Espina has contributed to methodology, investigation and formal analysis of the manuscript. María Luisa García has contributed to conceptualization, investigation, supervision, funding and review and editing of the manuscript. Ana Cristina Calpena has contributed to conceptualization, investigation, supervision, funding and review and editing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Acknowledgments

The authors would like to acknowledge Dr. Antoni Boix for its support during the in vivo studies. The authors would like to thank the electronic microscopy unit of the Scientific and Technological Center of the University of Barcelona for their technical support. The authors wish to acknowledge the support of the Spanish Ministry under the project PID2021-122187NB-C32 and the support of the Generalitat of Catalonia (2017SGR1447). ESL wants to acknowledge the requalification system grants.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2023.113678.

References

[1] K. Na-Bangchang, T. Plengsuriyakarn, J. Karbwang, The Role of Herbal Medicine in Cholangiocarcinoma Control: A Systematic Review, Planta Med 89 (1) (2023) 3–18.
[2] G. Grandi, G. Guariglia, F. Facchinetti, The role of combined oral contraceptives containing norgestimate for acne vulgaris treatment: a review, Eur J Contracept Reprod Heal Care 28 (3) (2023) 184–191.
[3] A. Kapuścińska, I. Nowak, Use of organic acids in acne and skin discolorations therapy, Postepy Hig Med Dosw 69 (2015) 374–383.
[4] E. Platsidaki, C. Dessinioti, Recent advances in understanding Propionibacterium acnes (Cutibacterium acnes) in acne, F1000Research 7 (0) (2018) 1953.
[5] L. Flowers, E.A. Grice, The Skin Microbiota: Balancing Risk and Reward, Cell Host Microbe 28 (2) (2020 12) 190–200.
[6] E.A. Grice, J.A. Segre, The skin microbiome, Nat Rev Microbiol 9 (4) (2011) 244–253.
[7] Ajay Bhatia, Jean-Francoise Maisonneuve, David H. Persing. Propinobacterium Acnes and chronic diseases. In: The Infectious Etiology of Chronic Diseases: Defining the Relationship, Enhancing the Research, and Mitigating the Effects: Workshop Summary. 2004.
[8] M. Otto, Staphylococcus epidermidis – the "accidental" pathogen, Nat Rev Microbiol 7 (8) (2009) 557–567.
[9] B. Dréno, E. Araviiskaia, D. Kerob, A. Andriessen, M. Anfilova, M. Arenbergerova, O.L. Forero Barrios, Z. Bukvić Mokos, M. Haedersdal, M.A. Hofmann, Z. Khamaysi, M. Kosmadaki, A. Lesiak, E. Roó, A. Zbranca-Toporas, M.C. Wiseman, S. Zimmo, L. Guerin, G. Fabbrocini, Nonprescription acne vulgaris treatments: Their role in our treatment armamentarium—An international panel discussion, J Cosmet Dermatol 19 (9) (2020) 2201–2211.
[10] H. Amiri, Essential oils composition and antioxidant properties of three thymus species, Evidence-based Complement Altern Med 2012 (2012) 1–8.
[11] M.F. Nagoor Meeran, G.S. Jagadeesh, P. Selvaraj, Thymol, a dietary monoterpene phenol abrogates mitochondrial dysfunction in β-adrenergic agonist induced myocardial infarcted rats by inhibiting oxidative stress, Chem Biol Interact 244 (2016 25) 159–168.
[12] R. Najafloo, M. Behyari, R. Imani, S. Nour, A mini-review of Thymol incorporated materials: Applications in antibacterial wound dressing, J Drug Deliv Sci Technol 60 (March) (2020), 101904.
[13] T.P. Pivetta, S. Simões, M.M. Araújo, T. Carvalho, C. Arruda, P.D. Marcato, Development of nanoparticles from natural lipids for topical delivery of thymol: Investigation of its anti-inflammatory properties, Colloids Surfaces B Biointerfaces 164 (2018) 281–290.
[14] S. Briganti, M. Picardo, Antioxidant activity, lipid peroxidation and skin diseases. What's new, J Eur Acad Dermatology Venereol 17 (6) (2003) 663–669.
[15] A. Wattanasatcha, S. Rengpipat, S. Wanichwecharungruang, Thymol nanospheres as an effective anti-bacterial agent, Int J Pharm 434 (1–2) (2012 15) 360–365.
[16] Q.D. Pham, S. Björklund, J. Engblom, D. Topgaard, E. Sparr, Chemical penetration enhancers in stratum corneum - Relation between molecular effects and barrier function, J Control Release 232 (2016) 175–187.
[17] C. Folle, A.M. Marqués, N. Díaz-Garrido, M. Espina, E. Sánchez-López, J. Badia, L. Baldoma, A.C. Calpena, M.L. García, Thymol-loaded PLGA nanoparticles: an efficient approach for acne treatment, J Nanobiotechnology 19 (1) (2021) 359.
[18] C. Folle, N. Díaz-Garrido, E. Sánchez-López, A.M. Marqués, J. Badia, L. Baldomà, M. Espina, A.C. Calpena, M.L. García, Surface-modified multifunctional thymol-loaded biodegradable nanoparticles for topical acne treatment, Pharmaceutics 13 (9) (2021).
[19] C. Martins, F. Sousa, F. Araújo, B. Sarmento, Functionalizing PLGA and PLGA Derivatives for Drug Delivery and Tissue Regeneration Applications, Adv Healthc Mater 7 (1) (2018).
[20] Z. Tang, X. Fan, Y. Chen, P. Gu, Ocular Nanomedicine, Adv Sci 9 (15) (2022) 1–36.
[21] S. Raman, A.A. Khan, S. Mahmood, Nose to brain delivery of selegiline loaded PLGA/lipid nanoparticles: Synthesis, characterisation and brain pharmacokinetics evaluation, J Drug Deliv Sci Technol 77 (2022), 103923.
[22] R. Gonzalez-Pizarro, P. Carvajal-Vidal, L. Halbault Bellowa, A.C. Calpena, M. Espina, M.L. García, In-situ forming gels containing fluorometholone-loaded polymeric nanoparticles for ocular inflammatory conditions, Colloids Surfaces B Biointerfaces 175 (2019) 365–374.
[23] X. Roig-soriano, E.B. Souto, F. Elmsmari, M.L. Garcia, M. Espina, F. Duran-sindreu, E. Sánchez-López, J.A. González Sánchez, Nanoparticles in Endodontics Disinfection: State of the Art, Pharmaceutics 14 (2022) 1519.
[24] E.B. Souto, J. Dias-Ferreira, A. López-Machado, M. Ettcheto, A. Cano, A. Camins Espuny, M. Espina, M.L. Garcia, E. Sánchez-López, Advanced Formulation Approaches for Ocular Drug Delivery: State-Of-The-Art and Recent Patents, Pharmaceutics 11 (9) (2019) 460.
[25] G. Amasya, O. Inal, C.T. Sengel-Turk, SLN enriched hydrogels for dermal application: Full factorial design study to estimate the relationship between composition and mechanical properties, Chem Phys Lipids 228 (2020), 104889.
[26] Vlaia L., Coneac G., Olariu I., Vlaia V., Lupuleasa D. Cellulose-Derivatives-Based Hydrogels as Vehicles for Dermal and Transdermal Drug Delivery. Emerg Concepts Anal Appl Hydrogels. (36355).
[27] Bachewad N.D., Jadhav N.C., Sonawane R.O. A REVIEW ON SOURCE, PHYSICOCHEMICAL PROPERTIES, GRADES, PREPARATION, EVALUATION, APPLICATIONS AND PATENTS OF CARBOPOL IN VARIOUS FORMULATIONS. Indo Am J Pharm Sci. 9(6):392–416.
[28] Unal S., Tekeli M., Dogan O., Aktas Y. Thermosensitive Pluronic® F127-Based in situ gel formulation containing nanoparticles for the sustained delivery of paclitaxel. Med Sci | Int Med J. 12(1):224.
[29] E. Sánchez López, G. Esteruelas, A. Ortiz, M. Espina, J. Prat, M. Muñoz, A. Cano, A. C. Calpena, M. Ettcheto, A. Camins, Z. Alsafi, E.B. Souto, M.L. García, M. Pujol, Dexibuprofen biodegradable nanoparticles: one step closer towards a better ocular interaction study, Nanomaterials. 10 (2020) 720.
[30] A. López-Machado, N. Díaz, A. Cano, M. Espina, J. Badía, L. Baldomà, A. C. Calpena, M. Biancardi, E.B. Souto, M.L. García, E. Sánchez-López, Development of topical eye-drops of lactoferrin-loaded biodegradable nanoparticles for the treatment of anterior segment inflammatory processes, Int J Pharm 609 (2021), 121188.
[31] J. Suñer-Carbó, A. Boix-Montañés, L. Halbaut-Bellowa, N. Velázquez-Carralero, J. Zamarbide-Ledesma, N. Bozal-de-Febrer, A.C. Calpena-Campmany, Skin permeation of econazole nitrate formulated in an enhanced hydrophilic multiple emulsion, Mycoses 60 (3) (2017) 166–177.
[32] P. Carvajal-Vidal, R. González-Pizarro, C. Araya, M. Espina, L. Halbaut, I. Gómez de Aranda, M.L. García, A.C. Calpena, Nanostructured lipid carriers loaded with Halobetasol propionate for topical treatment of inflammation: Development, characterization, biopharmaceutical behavior and therapeutic efficacy of gel dosage forms, Int J Pharm 585 (2020), 119480.
[33] S. Messager, A.C. Hann, P.A. Goddard, P.W. Dettmar, J.Y. Maillard, Use of the "ex vivo" test to study long-term bacterial survival on human skin and their sensitivity to antisepsis, J Appl Microbiol 97 (6) (2004) 1149–1160.
[34] A.P. MacGowan, M. Wootton, A.J. Hedges, K.E. Bowker, H.A. Holt, D.S. Reeves, A new time-kill method of assessing the relative efficacy of antimicrobial agents alone and in combination developed using a representative β-lactam, aminoglycoside and fluoroquinolone, J Antimicrob Chemother 38 (2) (1996) 193–203.
[35] P. Gava Mazzola, T.C. Vessoni Penna, A.M. Alzira, Determination of decimal reduction time (D value) of chemical agents used in hospitals for disinfection purposes, BMC Infect Dis 3 (1) (2003) 10.
[36] D. Mazia, G. Schatten, W. Sale, Adhesion of cells to surfaces coated with polylysine: Applications to electron microscopy. J Cell Biol 66 (1) (1975) 198–200.
[37] N. Diaz-Garrido, M.J. Fábrega, R. Vera, R. Giménez, J. Badia, L. Baldomà, Membrane vesicles from the probiotic Nissle 1917 and gut resident Escherichia coli strains distinctly modulate human dendritic cells and subsequent T cell responses, J Funct Foods 61 (2019), 103495.
[38] Tong Wu QZGHZBJBHXCC. A chlorogenic acid-chitosan complex bifunctional coating for improving osteogenesis differentiation and bactericidal properties of zirconia implants. Colloids Surfaces B Biointerfaces. 230.
[39] Issei Takeuchi, YHTOCTKMKSKM. Chitosan-coated PLGA nanoparticles for transcutaneous immunization: Skin distribution in lysozyme-sensitized mice, Colloids Surfaces B Biointerfaces 220 (2022), 112916.

[40] Y.A. Aguilar-López, L. Villafuerte-Robles, Functional Performance of Chitosan/Carbopol 974P NF Matrices in Captopril Tablets, J Pharm (2016) 1–9.
[41] E. Sánchez-López, M. Ettcheto, M.A. Egea, M. Espina, A.C. Calpena, J. Folch, A. Camins, M.L. García, New potential strategies for Alzheimer's disease prevention: pegylated biodegradable dexibuprofen nanospheres administration to APPswe/PS1dE9, Nanomedicine Nanotechnology, Biol Med 13 (3) (2017) 1171–1182.
[42] E. Sánchez-López, M. Ettcheto, M.A. Egea, M. Espina, A. Cano, A.C. Calpena, A. Camins, N. Carmona, A.M. Silva, E.B. Souto, M.L. García, Memantine loaded PLGA PEGylated nanoparticles for Alzheimer's disease: in vitro and in vivo characterization, J Nanobiotechnology 16 (32) (2018) 1–16.
[43] T.E. Rajapaksa, M. Stover-Hamer, X. Fernandez, H.A. Eckelhoefer, D.D. Lo, Claudin 4-targeted protein incorporated into PLGA nanoparticles can mediate M cell targeted delivery, J Control release 142 (2) (2010) 196–205.
[44] I. Manou, L. Bouillard, M.J. Devleeschouwer, A.O. Barel, Evaluation of the preservative properties of Thymus vulgaris essential oil in topically applied formulations under a challenge test, J Appl Microbiol 84 (3) (1998) 368–376.
[45] M. Mallandrich, F. Fernández-Campos, B. Clares, L. Halbaut, C. Alonso, L. Coderch, M.L. Garduño-Ramírez, B. Andrade, A. Del Pozo, M.E. Lane, A.C. Calpena, Developing Transdermal Applications of Ketorolac Tromethamine Entrapped in Stimuli Sensitive Block Copolymer Hydrogels, Pharm Res 34 (8) (2017) 1728–1740.
[46] M.E. Parente, A. Ochoa Andrade, G. Ares, F. Russo, A. Jiménez-Kairuz, Bioadhesive hydrogels for cosmetic applications, Int J Cosmet Sci 37 (5) (2015) 511–518.
[47] J. Russo, J. Fiegel, N.K. Brogden, Rheological and drug delivery characteristics of poloxamer-based diclofenac sodium formulations for chronic wound site analgesia, Pharmaceutics 12 (12) (2020) 1–18.
[48] A.C. Calpena, B. Clares, Fernández F. Technological, biopharmaceutical and pharmacokinetic advances: New formulations of application on the skin and oral mucosa, in: D. Muñoz-Torrero (Ed.), Recent Advances in Pharmaceutical Sciences, Transworld Research Network, Kerala, India, 2011, pp. 175–198.
[49] H.N. Ho, T.G. Le, T.T.T. Dao, T.H. Le, T.T.H. Dinh, D.H. Nguyen, T.C. Tran, C. N. Nguyen, Development of Itraconazole-Loaded Polymeric Nanoparticle Dermal Gel for Enhanced Antifungal Efficacy, J Nanomater (2020) 1–11.
[50] Moussaoui S.El, Fern F., Alonso C., Lim D., Halbaut L., Garduño-ramirez M.L., et al. Ablation, or Surgical Removal in Condyloma Acuminata.
[51] Kazak M., Donmez N., Bahadori F., Yenigun V.B., Kocyigit A. Estudio preliminar in vitro de la citotoxicidad de resinas compuestas caducadas y no caducadas. Odovtos - Int J Dent Sci. 22(3):123–134.