



Hyaluronic acid as a key modulator of physicochemical, thermal and rheological properties in thermosensitive composite hydrogels for ocular drug delivery

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ABSTRACT

This study investigates the role of hyaluronic acid (HA) in modulating the structure-property relationships of an injectable composite drug delivery system (c-DDS) for ocular drug administration. The c-DDS was designed to behave as a viscous fluid at room temperature, enabling intravitreal injection, and to undergo in situ gelation at physiological ocular temperature. The system consists of a thermosensitive poloxamer-based hydrogel embedding biodegradable poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles (NPs). HA was incorporated within the hydrogel and as a surface coating on PLGA NPs to modulate the physicochemical properties of the system and control NP mobility. The hydrogels maintained their rheological properties up to four weeks, also following intravitreal injection through a fine-gauge needle and autoclave sterilization. Swelling and thermal analyses highlighted the contribution of each component to the overall behaviour of the system. Multiple particle tracking (MPT) analysis of NP diffusion within the hydrogels demonstrated a crucial role of HA in tuning NP mobility. In vitro studies using ARPE-19 cells confirmed the biocompatibility of the c-DDS and showed effective NPs internalization. Overall, this preliminary study lays a formulation framework for HA-modulated injectable composite hydrogels. This also upholds their further development as ocular delivery platforms for the loading of diverse therapeutic agents, ranging from hydrophilic to lipophilic and from low- to high-molecular weight, along with the subsequent evaluation of their biological implications.

1. Introduction

Visual impairment is a major global health concern, with over 2.2 billion people affected by ocular diseases such as diabetic retinopathy, age-related macular degeneration, and glaucoma (Swenor et al., 2020). Their treatment is hindered by ocular barriers, such as pre-corneal, corneal, blood-ocular, and blood-retinal, which severely limit drug

permeation. Blinking, tear production, nasolacrimal drainage and enzymatic degradation further limit drug permeation, retention, and biodistribution (Bisht et al., 2018). Intravitreal injection is so far the most effective route for targeting the posterior eye segment, as it allows the direct drug deposition into the vitreous body. However, this invasive procedure is related to serious risks, including retinal detachment, infection and hemorrhage (Zhu et al., 2023). Also, since posterior eye

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diseases are mostly chronic, repeated injections are needed, causing cumulative side effects and high healthcare costs (Qi et al., 2023).

To address these setbacks, recent research has focused on innovative drug delivery systems (DDSs) enabling controlled and local drug delivery, thereby reducing the frequency of administration and the adverse effects associated with the injection (Xie et al., 2015b). Microparticles (MPs), nanoparticles (NPs), polymeric implants, and *in situ* forming hydrogels have shown promising results as ocular DDSs (Baranowski et al., 2014) (Shah et al., 2010). *In situ* forming hydrogels are hydrophilic polymeric systems that undergo sol–gel transitions in response to stimuli such as temperature, pH, or ionic strength (Malta et al., 2023) (Rasool et al., 2019). Among them, thermosensitive hydrogels are particularly attractive for tissue engineering, regenerative medicine, wound healing, and controlled drug delivery. They are strongly relevant for intravitreal administration because they remain liquid at room temperature and rapidly gel at physiological temperatures, creating a localized drug depot (Wu et al., 2019).

Poloxamers (Pluronic®) are thermosensitive copolymers composed of alternate poly(ethylene oxide) and poly(propylene oxide) segments, approved by Food and Drug Administration (FDA) and generally recognised as safe (GRAS) excipients. Their amphiphilic nature enables micellization above the critical micelle concentration (CMC) (Mfoafo et al., 2022). Under these conditions, increasing the temperature gradually reduces micellar hydrophilicity, promoting the rearrangement of micelles into more complex structures and ultimately leading to the reversible gelation of the poloxamer solution at the critical micellization temperature (CMT) (Mfoafo et al., 2022). Mixtures of different poloxamers are attractive for ocular drug delivery since their CMT is close to the physiological value and owing to their concentration-dependent viscosity, optical clarity, shear-thinning behavior, and ocular biocompatibility. However, their limited mechanical properties and mucoadhesiveness may restrict their performance (Diaz-Salmeron et al., 2021). Thus, to overcome these shortcomings, poloxamers are often combined with other polymers, such as hydroxypropyl methylcellulose (HPMC), showing good ocular tolerability *in vivo* (Taheri et al., 2022). Similarly, we have produced gels composed of poloxamers and hyaluronic acid (HA), with tailorable thermosensitivity and enhanced mechanical properties (Villapiano et al., 2024). Nonetheless, their relatively fast drug release may limit their suitability for sustained intravitreal delivery.

Considering this, the incorporation of NPs into hydrogel matrices can create composite systems (cDDS) in which NPs act as secondary reservoirs while the hydrogel provides injectability, spatial localization, and an additional diffusional barrier. This cDDS architecture can be tuned by adjusting NP properties and hydrogel composition, aiming for an improved nanocarrier local retention, reduced aggregation and a further control parameter over drug release reducing initial burst release commonly associated with NP-based formulations (Lv et al., 2025).

Thus, several thermosensitive cDDS have been reported for intravitreal delivery (Ottonelli et al., 2022), (Zhang et al., 2021), (Wen et al., 2025), (Sapino et al., 2019) (du Toit et al., 2021). Besides, using the hydrogel as a nanocarriers cargo may also reduce the dependence of therapy effectiveness on the pathological state of the patient. Indeed, disease-related declining of vitreous body viscoelasticity may facilitate particle migration within the vitreous space may occur, possibly impairing patient *visus* (Levin & Cohen, 2021). Altogether, for a rational cDDS design for intravitreal administration, NPs diffusion within the hydrogel and the vitreous body is a crucial parameter.

Thus, in this study, we designed an injectable cDDS by incorporating HA-decorated NPs based on polylactic-co-glycolic acid (PLGA) into a poloxamer/HA thermosensitive hydrogel intended for intravitreal administration. Indeed, we hypothesized that by using the same polysaccharide both within the hydrogels and as a surface coating of the embedded PLGA-based NPs could modulate the physicochemical properties of the system and influence NP mobility in the matrix. Since HA is a natural component of the vitreous humor, its use for NP coating may

also affect particle transport in the vitreous environment. PLGA was selected as a biodegradable and biocompatible copolymer, widely used in drug delivery. In physiological conditions, it hydrolyzes into lactic acid and glycolic acid, which are further metabolized via normal metabolic pathways. Thus, the degradation products are biocompatible and safe for ocular applications, supporting the use of PLGA for ophthalmic drug delivery systems. (Su et al., 2021) (Biswas et al., 2024)

The cDDS was evaluated for key translational requirements, including storage stability, injectability, sterilization feasibility, swelling, thermal properties and biocompatibility. Furthermore, multiple particle tracking (MPT) microrheology was employed to assess how gel composition impacts NP mobility within the hydrogels and in a simulated vitreous body (SVB). Finally, ARPE-19 cells were used to evaluate *in vitro* cDDS biocompatibility and NP internalization.

2. Materials and methods

2.1. Materials

Altergon Italia s.r.l. (Morra De Sanctis (AV), Italy) kindly provided HA with a molecular weight of 830 kDa. Poloxamers (PEOa–P–POb–PEOa), F127 (a = 100 and b = 65) and F68 (a = 76 and b = 29), equimolar uncapped poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG502H, Mw 7000 – 17000). Rhodamine B-labelled PLGA (lactide: glycolide 50:50, Mw 10000 – 30000) (PLGA RHOD), acetone, dibasic sodium phosphate (Na₂HPO₄), potassium chloride (KCl) and sodium chloride (NaCl) were supplied by Sigma-Aldrich (St. Louis, MO, Milan, Italy). Dialysis membranes (Biotech RC tubing, MCWO: 3.5–5 kDa) were purchased from Spectra/Por®. For cell culture experiments, Arising retinal pigment epithelia (ARPE-19) (CRL-2302, ATCC®) cells were supplied by LGC Standards S.r.l., (Milan, Italy), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), sodium pyruvate, glutamine and antibiotic mixture (0.02 mg/mL streptomycin and 0.02 IU/mL penicillin) were purchased from Euroclone SpA (Pero (MI), Italy). All chemicals and media were used as received.

2.2. Hydrogels production

Based on a previously reported procedure, three different hydrogel formulations were prepared: P3 0.1%, P3 1%, and P4 0.1%. Briefly, the two poloxamers, F127 and F68, were dispersed in filtered water (0.2 µm filter) and continuously stirred at 250–300 rpm in an ice bath at 4°C until a clear solution was obtained. Subsequently, the samples were stored overnight at 4°C. HA was then added, and stirred at 300 rpm in an ice bath until the solution became clear. Finally, the dispersion was stored again at 4°C until complete solubilization was achieved. The composition of the formulations is summarized in Table 1.

2.3. Rheological characterization of hydrogel mechanical properties

The viscoelastic properties of the hydrogels were investigated in an earlier study by small-amplitude oscillatory shear experiments using a rotational rheometer (Malvern-Kinexus) (Villapiano et al., 2024). Here we assessed the stability of their mechanical properties over time, after autoclave sterilization and following injection through an intravitreal injection needle (27G). In all cases, the rheological measurements were performed by small-amplitude oscillatory shear experiments using a

Table 1
Composition of the hydrogels.

Formulation	F68 (% w/v)	F127 (% w/v)	HA (% w/v)
P3 0.1%	21.43	21.43	0.1
P3 1%	21.43	21.43	1
P4 0.1%	30	30	0.1

rotational rheometer (Malvern-Kinexus) equipped with a passive heat exchanger temperature-control system (KNX2500), with a cone/plate geometry (CP4/40) as the measurement system. Thus, hydrogel elastic (G') and viscous moduli (G'') were measured, at 37°C, as a function of frequency, in 0.1-10 Hz frequency range, and within their linear viscoelastic region (LVER).

For stability studies, the measurements were conducted at different time points (0, 7, 14, 21, and 28 days) on samples stored at 4°C. To assess potential changes in the rheological properties of the hydrogels following injection, they were injected directly onto the rheometer plate through the needle (27G) of a 1 mL syringe for intravitreal injection. The hydrogels were sterilized by autoclaving (CertoClav Sterilizer GmbH, A-4060 Leonding, Austria) by a standard cycle of 15 minutes at 121°C and 2 atm.

Furthermore, viscosity measurements were performed at 25°C, using a rotational rheometer (Kinexus, Malvern Instruments, Malvern, PA, USA), equipped with a KNX2500 passive heat exchanger temperature-control system. A cone-and-plate geometry (CP4/40) was used and a controlled shear rate sweep from 0.01 to 1000 s⁻¹ was applied to the samples. All experiments were carried out in triplicate.

2.4. Evaluation of injection force

To assess the force required to inject the hydrogels through an intravitreal injection needle (27G, 13 mm), a predictive model for non-Newtonian fluids was employed (Rheosense.com, 2026). The experimental data obtained from the viscosity studies were used to determine the model coefficients a and b by fitting the relationship between shear rate ($\dot{\gamma}$) and shear stress (σ) using the following quadratic equation 1:

$$\dot{\gamma}(\sigma) = a\sigma^2 + b\sigma \quad (1)$$

Then, coefficients A and B were determined by means of equations 2 and 3, respectively:

$$A = \frac{a}{5} \quad (2)$$

$$B = \frac{b}{4} \quad (3)$$

These parameters, together with coefficient C (Eq. 4), were then used to determine wall shear stress at the inner surface of the needle (σ_w) (MPa) (Eq.5).

$$C = \frac{-Q}{\pi R_n^3} \quad (4)$$

$$\sigma_w = \frac{-B + \sqrt{B^2 - 4AC}}{2A} \quad (5)$$

where Q is the volumetric flow rate (mm³s⁻¹) and R_n is the inner radius of the needle (m).

Using this σ_w value, together with the specifications of the injection system (27G × 1/2" needle and 1 mL plastic syringe), the injection force was then calculated according to equation 6:

$$F_v \cong 2\pi l_n \sigma_w \frac{R_p^2}{R_n} \quad (6)$$

where F_v is the injection force (N), l_n the needle length (m) and R_p (m) is the radius of the piston of the syringe.

2.5. Measurement of swelling pressure

The swelling pressure of the hydrogels was assessed by evaluating their weight changes in time after being placed in a dialysis membrane (Biotech RC tubing, MCWO: 3.5-5 kDa) in phosphate buffer saline (PBS) and in SVB (Laradji et al., 2020). Specifically, 500 µL of each hydrogel

were placed in 20 mL of medium at 37°C. At predeterminate time points (0,1,2,4,6,22,24,28,46,48,70 and 72h) the dialysis membranes were dried and weighted. The swelling ratio (SR) was calculated by equation 7:

$$SR = \frac{W_s - W_d}{W_d} \quad (7)$$

where W_s and W_d are the weight of the membranes containing swollen and dried hydrogels, respectively (mg).

One-way ANOVA was used to evaluate the significance of the differences among the various hydrogel formulations and, for each hydrogel, between the two media. A p value ≤ 0.05 was considered to indicate a statistically significant difference. All the experiments were performed at least in biological and technical triplicate.

2.6. Thermal analyses

Thermogravimetric analyses have been performed on freeze-dried gels (P3 0.1%, P3 1% and P4 0.1%) by TGA (Discovery™ TGA55, TA Instruments, Waters™, New Castle, DE, USA) to determine the presence/absence of residual solvents in the samples due to the preparation procedures, to assess their thermal resistance, to analyze their thermal degradation process, to examine any interaction eventually occurring among the materials. The TGA analyses have been performed in dynamic mode, from 25°C to 600°C, using a heating rate of 10°C/min. However, some tests were extended up to 1000°C if the samples had not reached complete degradation at 600°C. The rationale for testing the samples thermo-gravimetrically up to temperatures well above physiological ones was to analyze up to which temperature the materials could be considered stable, that is, to provide a more complete characterization of them until their complete thermal degradation was achieved. Three samples of each composition of lyophilized gel, weighing between 10 and 15 mg placed in alumina crucibles, have been analyzed to check the repeatability of results. For comparison purposes, pure formulations P3 and P4, i.e. without HA, were also analyzed in TGA analyzer, using the same procedure previously described.

The gels in the hydrated state have been analyzed also in isothermal conditions to examine the evaporation process taking place (up to 3 h) at 60°C. The time after which a constant weight is reached and the residual weight have been calculated on at least three specimens for each formulation to check the repeatability of results, averaging these results.

The results of these tests were expressed as a change in mass, normalized to the initial weight of each sample, as a function of temperature (for dynamic tests) or time (for isothermal tests).

2.7. Production of PLGA- based NPs

Biodegradable bare NPs (B-NPs), based on FDA-approved polyesters PLGA, were prepared using nanoprecipitation technique followed by solvent evaporation (Serri et al., 2019). Briefly, an internal aqueous phase (W_0) consisting of 125 µL of double distilled water (DDW) was mixed by sonication (5 min, 4°C, Branson 2800 ultrasonic bath, 100% power, operating at 40 kHz $\pm$ 6%) with an organic phase composed of 1.25 mL of a PLGA:F68:F127 (mass ratio 1:0.5:0.5) solution in acetone (total polymer concentration: 2% w/v). The resulting mixture was forced through a syringe needle (22 G) at a 333.3 µL/min flow rate by a syringe pump into 10 mL of an external aqueous phase (W_1), under magnetic stirring, containing poloxamers as surface-active agents (0.05% w/v; F68:F127 weight ratio 1:1), in the presence or absence of HA (0.75 mg/mL). Acetone was finally evaporated by a rotary evaporator at 37°C and the obtained suspension was washed twice by centrifugation at 10,000 rpm first for 10 minutes, then for 20 minutes (DLAB D1524R, China).

Fluorescent PLGA NPs were prepared using Rhodamine B-labelled PLGA, in which Rhodamine B is chemically bound to the PLGA

backbone. Rhodamine B was used as a fluorescent tracer to enable NP visualization during MPT and cellular internalization experiments. A 1:9 weight ratio with non-fluorescent PLGA was used. The composition and corresponding acronyms of the NP formulations are summarized in Table 2.

2.8. Particle technological characterization

Intensity-average diameters, polydispersity index (PDI) and ζ potentials of NPs were determined via dynamic light scattering (DLS) measurements with a Zetasizer Nano (Malvern Instruments, Malvern, UK). For particle size measurements, NPs were suspended in ultrapure water.

The yield of NPs was gravimetrically determined from the NP mass recovered after freeze-drying (HetoPowerDry PL6000 Freeze Dryer, Thermo Electron Corp., USA; -60°C , 0.73 hPa, 24 h) and expressed as the percentage of the obtained mass of particles with respect to their initial mass.

2.9. Preparation of the e composite drug delivery system (cDDS)

The cDDS were prepared by incorporating an appropriate amount of NPs into each hydrogel. To ensure optimal mixing between the NPs and the hydrogel, the NPs were combined with the cold hydrogel and thoroughly vortexed. Fluorescent NPs were used for transport studies, as described in Section 2.10, whereas non-fluorescent NPs were employed for UV-Vis studies aimed at assessing the optical transparency of the cDDS (see in supplementary data).

2.10. Transport studies of NPs

Diffusion studies of fluorescent NPs were studied both in formulated gels (P3 0.1%, P3 1% and P4 0.1%) and in simulated vitreous body (SVB). The SVB was prepared by adding agar 0.95 (mg/mL) and HA (0.70 mg/mL) to boiling water (Thakur et al., 2020). After complete dissolution of agar and HA, the preparation was stored at room temperature overnight. The transport of NPs inside the gels was analyzed by multiple particle tracking (MPT), which allows monitoring the movement of single particles in real time, providing precise information on the dynamics and interactions between NPs and the gel matrix. In the MPT experiments, fluorescent NPs (PP and PP HA) were incorporated into gels and SVB at a dilution ratio of 1:10. The samples were loaded into rectangular cross-section capillaries (0.4×4 mm) for image acquisition. Prior to image acquisition, the samples were carefully mounted on the microscope holder and allowed to equilibrate at 37°C for 10 min to ensure gel relaxation. The setup was placed on an active optical table to reduce the effect of vibrations. The motion of NPs embedded in at least 10 different regions of each sample (formulated gels and SVB) was recorded using an inverted fluorescence microscope (Olympus IX81; Olympus) equipped with a $60\times$ water-immersion objective (N.A. = 1.20) and a Hamamatsu ORCA-Flash 2.8 CMOS camera (Hamamatsu). For formulated gels, a $1.6\times$ optical lens was additionally employed. Image sequences of 25 s were acquired at a frame rate of 4 fps for formulated gels, whereas for the SVB, image sequences of 20 s were acquired at 10 fps.

Particle trajectories were reconstructed using a custom Matlab al-

gorithm in two steps: localization of beads in each frame, followed by trajectory linking. Positions were determined from intensity-weighted centroids and compared across consecutive frames under the assumption that the closest positions correspond to the same particle (proximity principle). Mean squared displacements (MSDs) were then calculated from the reconstructed trajectories according to the following equation 8:

$$\langle \Delta r^2(t) \rangle = \langle [x(t - \tau) - x(t)]^2 + [y(t - \tau) - y(t)]^2 \rangle \quad (8)$$

where $\langle \rangle$ angular brackets mean time average, τ is the time scale, and t is the elapsed time. The average MSD of all NPs belonging to the same region was used to derive the viscosity of the sample by applying the following different approaches. The MSDs of individual particles were fitted with a power law (Eq.9):

$$\langle \Delta r^2(t) \rangle = 2nDt^\alpha \quad (9)$$

where n is the dimensionality (equal to 2 in this case). Only trajectories with $R^2 > 0.7$ and $0.2 < \alpha < 1.05$ were retained and the viscosity of gels and SVB was determined from the average MSD of NPs within the same region, using one of the two approaches described below.

When the scaling exponent α is close to 1 ($\alpha > 0.95$), gels behave as purely viscous fluid, and the diffusion coefficient D is linked to gel viscosity through the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta a} \quad (10)$$

Where η denotes viscosity, a is the particle radius, k_B is Boltzmann's constant, and T is the absolute temperature (thus, $k_B T$ represents the thermal energy).

If the scaling exponent α is less than 1 ($\alpha < 0.95$), the sample exhibits viscoelastic behavior. In this case, a modified algebraic form of the generalized Stokes-Einstein equation allows the MSD to be converted into the time-dependent creep compliance $J(t)$:

$$J(t) = \frac{3\pi a}{2k_B T} \langle \Delta r^2(t) \rangle \quad (11)$$

Finally, within the Maxwell model for viscoelastic behavior, creep compliance $J(t)$ can be related to both gel viscosity η and the elastic modulus G :

$$J(t) = \frac{1}{G} + \frac{1}{\eta} t \quad (12)$$

Statistical analyses were performed on MSD values at 4 s, creep compliance at 4 s, and viscosity measurements. Data normality was assessed using the Shapiro-Wilk test ($p < 0.05$ indicating deviation from normality). For normally distributed datasets, comparisons were carried out with an unpaired Student's t -test, whereas non-normal data were analyzed using the nonparametric Kruskal-Wallis test (Tables S1-S3). Statistical significance was defined as $p < 0.05$.

2.11. In vitro studies

2.11.1. Cell culture

ARPE-19 cells were cultured in DMEM/F-12 supplemented with 10% heat-inactivated FBS, 10 mM sodium pyruvate, 2 mM glutamine, and an

Table 2
Composition of the different NPs formulations.

Formulation	W ₀	Organic phase				W ₁		
		PLGA (% w/v)	PLGA RHOD (% w/v)	F68 (% w/v)	F127 (% w/v)	HA (% w/v)	F68 (% w/v)	F127 (% w/v)
B-PP	125	1	-	0.5	0.5	-	0.025	0.025
B-PP-HA	125	1	-	0.5	0.5	0.075	0.025	0.025
PP	125	0.9	0.1	0.5	0.5	-	0.025	0.025
PP HA	125	0.9	0.1	0.5	0.5	0.075	0.025	0.025

antibiotic mixture (0.02 mg/mL streptomycin and 0.02 IU/mL penicillin) at 37°C in a 5% CO₂ humidified atmosphere.

2.11.2. Cell viability assay

ARPE-19 cells were seeded in 24-well plates, at a density of $\sim 4 \times 10^4$ cells/well 24 h before treatment and incubated at 37 °C and 5% CO₂. To assess hydrogel biocompatibility, after 24 h of culture, the medium was replaced with fresh medium containing P3 1%, P4 0.1%, or P3 0.1% hydrogels at 1:10, 1:20, 1:40, or 1:80 v/v dilutions. Cells incubated with DMEM/F-12 alone were used as a negative control.

Similarly, NP cytotoxicity was assessed by treating ARPE-19 cells with different dilutions (160, 80, 40, and 20 ng/μL) of both B-PP and B-PP-HA NPs. Furthermore, the cytotoxicity of the various cDDSs was assessed using the highest concentrations of both components, namely a 1:10 dilution of the hydrogels and 160 ng/μL of NPs. Cell viability was evaluated by MTT assay (3-[4-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue] in standard culture conditions for 1 h and formazan crystals were dissolved in Isopropyl alcohol. The reduction of MTT to formazan was measured at 570 nm with a microplate reader (Agilent BioTek Synergy H1 multimode reader), providing an indirect indication of cell viability. The assay was performed in technical and biological triplicate.

Intracellular production of reactive oxygen species in ARPE-19 cells after 48 h treatment with cDDSs formulated at the highest concentrations of both components was measured using CellROX® Green Oxidative Stress Reagent according to the manufacturer's instructions (ThermoFisher Scientific). Green photostable fluorescence was determined with a microplate reader (Agilent BioTek Synergy H1 multimode reader). Cells treated for 10 min with 0.7% H₂O₂ before adding CellROX green were used as positive control.

2.11.3. NP in vitro internalisation

ARPE-19 cells were seeded and incubated in a 24-well plate in the previously described culture conditions. Cultured cells were treated with fluorescent PP or PP HA NPs, administering different concentrations (40 ng/μL, 20 ng/μL, 10 ng/μL) to determine a possible dose-dependent effect on internalisation. In addition, different incubation times (1 hour, 3 hours, 5 hours, 24 hours) were tested to optimise the internalisation process. After exposure, cells were fixed with 4% paraformaldehyde at room temperature for 20 min and then permeabilized with 0.1% Triton-X100 at room temperature for 10 min. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, cells were washed with PBS between each step. The cells were observed using a fluorescent microscopy (Mateo Digital Microscope, Leica) with a high-resolution objective, typically 20x, to monitor the internalisation of NPs in the treated cells.

2.12. Statistical analysis

All the statistical analysis were performed using Graph Pad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA). Two-way ANOVA was used to evaluate the significance of the differences of one variable in more than two groups. Unpaired two-tailed *t* test was used for two group comparison. Tukey's multiple comparisons test with Bonferroni post hoc correction was used to assess the significance between each treatment group and the control group. $p \leq 0.05$ was considered to indicate a statistically significant difference. All the experiments were performed at least in biological and technical triplicate.

3. Results

3.1. Rheological characterization of hydrogel mechanical properties

The hydrogel composition was optimized in our previous study with respect to its rheological properties, aiming to achieve predominantly viscous behavior at room temperature to ensure syringeability through

needles suitable for intravitreal administration (27G), while retaining the ability to undergo gelation at temperatures close to physiological conditions (Villapiano et al., 2024). More specifically, formulations containing HA concentrations higher than 1% w/v were excluded because they exhibited excessive viscosity, making them unsuitable for ocular injection, even at temperatures below 4°C. Conversely, formulations containing less than 21.43% w/v poloxamer remained in a liquid state at temperatures above 37°C and/or displayed excessively long gelation times. Therefore, among the formulations investigated, only three met the required criteria. Their compositions are reported in Table 1. These formulations exhibited gelation near body temperature while maintaining sufficiently short gelation times to enable rapid and practical ocular administration.

The results of rheological analyses of the hydrogels are shown in Figs. 1, which illustrates the rheological behavior of the hydrogels at different storage times at 4°C. The viscoelastic properties remained substantially unchanged throughout the entire storage period for all formulations. Notably, all hydrogels retained the characteristic rheological behavior typical of a gel at 37°C, with a predominant storage modulus (G') over the loss modulus (G'') in the entire frequency range tested, with both viscoelastic moduli quite constant with frequency. The same results were obtained following sterilization by autoclaving and injection through a 27G needle suitable for intravitreal administration, with mechanical spectra remaining essentially superimposable to the corresponding spectra recorded at time 0.

3.2. Evaluation of injection force

Since all hydrogels were successfully injected at 25°C through a 27G needle, suitable for intravitreal administration, the force required to perform the injection was also estimated. To inject 100 μL of each hydrogel formulation at a constant flow rate of 2.5 μL/s, the force applied to the plunger was found to be approximately 0.16, 0.41 and 0.79 N for P3 0.1, P3 1, and P4 0.1, respectively.

3.3. Measurement of swelling pressure

The water absorption capacity of the hydrogels was assessed through swelling experiments. Hydrogels were weighed at predetermined time intervals after immersion in PBS and SVB to monitor their swelling behaviour, as shown in Fig. 2. Maximum swelling was observed at 22 hours for P3 0.1%, 28 hours for P3 1%, and 24 hours for P4 0.1%, with all formulations reaching a plateau within 70 hours. The swelling ratios (SR) were found to be 5.11 ± 0.73 for P3 0.1%, 4.94 ± 0.31 for P3 1%, and 4.28 ± 1.37 for P4 0.1%, highlighting a slight influence of the formulation composition on water uptake although, at the concentration used in this study, the differences were not statistically significant.

3.4. Thermal analyses

Figs. 3 and Tables 3–4 summarize the results of thermogravimetric analysis (TGA) performed on both hydrated and lyophilized hydrogel samples. These analyses were conducted on formulations P3 0.1%, P3 1%, and P4 0.1%, as well as on P3 and P4, which represent the hydrogel platforms without hyaluronic acid (HA) and serve as controls to assess the impact of HA on thermal properties.

Figs. 3A–B show the dynamic TGA curves of the lyophilized gels. The incorporation of HA clearly shifted the onset temperature of thermal degradation to higher values in these samples, with the shift becoming more pronounced as HA content increased. Table 3 provides the corresponding onset and end temperatures, derivative thermogravimetry (DTG) values, and DTG peak temperatures obtained from the dynamic TGA analyses.

Figs. 3C–D display the isothermal TGA curves of the hydrated gels. Consistent with the results observed for the lyophilized samples, the presence of HA delayed the evaporation process and reduced its rate,

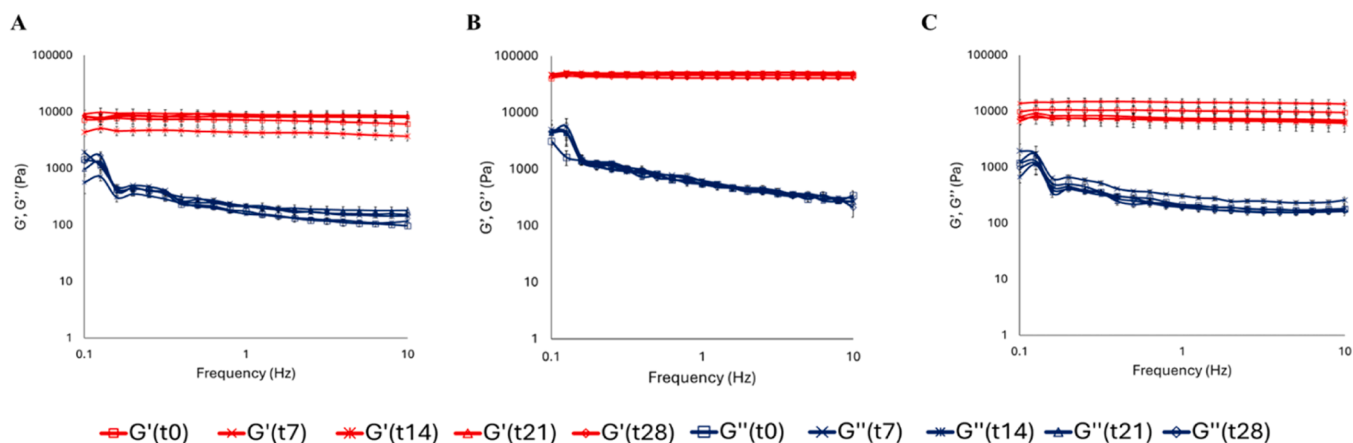


Fig. 1. Stability studies: rheological spectra of P3 0.1% (a), P3 1% (b) and P4 0.1% (c) at 37°C, at different time points during the storage period at 4°C.

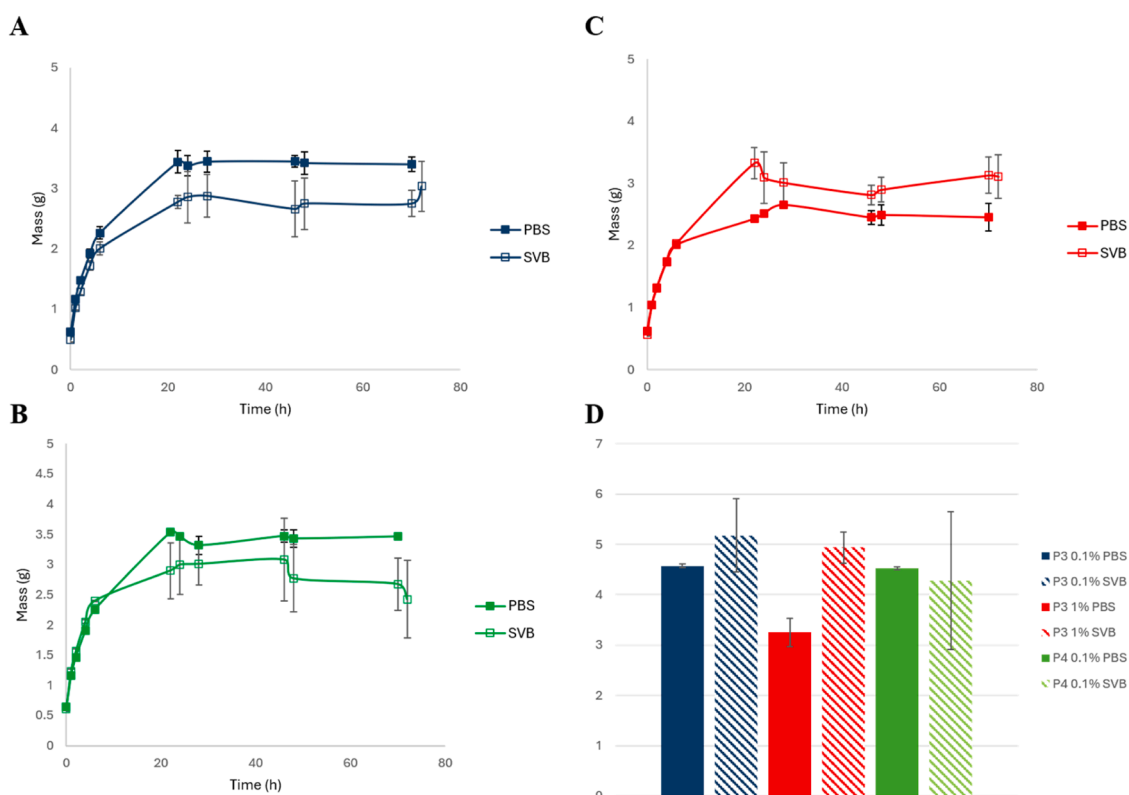


Fig. 2. Swelling behaviour of hydrogel at 37°C: swelling curve of P3 0.1% (A), P3 1% (B), P4 0.1% (C) in PBS and SVB; swelling ratio of P3 0.1%, P3 1% and P4 0.1% in PBS and SVB.

regardless of the specific formulation tested. Moreover, this effect became more significant with increasing HA content. The corresponding end time and weight loss of the hydrogels determined during isothermal analyses are summarized in Table 4.

3.5. Particle technological characterization

Two different NPs, B-PP and B-PP-HA were prepared according to a previously optimized procedure (Della Sala et al., 2022). Physico-chemical characterization showed that B-PP NPs had a mean diameter of 137.4 ± 2.1 nm, a low polydispersity index (PDI) (0.05 ± 0.01), and a negative zeta potential (25.84 ± 2.08 mV). While B-PP-HA NPs exhibited a larger mean diameter (166.7 ± 0.7 nm), a higher PDI (0.16 ± 0.02) and a more negative zeta potential (-48.69 ± 1.18 mV). Similarly, the

two fluorescent PLGA-based NPs formulations, either with or without HA coating, referred to as PP and PP HA respectively, were characterized. The PP NPs showed a mean diameter of 153 ± 1 nm, a PDI of 0.11 ± 0.02 , and a zeta potential of -34.9 ± 1.2 mV. In contrast, PP HA NPs the mean diameter increased to 232 ± 4 nm, while the PDI and zeta potential reached 0.29 ± 0.01 and -60.2 ± 0.7 mV.

3.6. Transport studies

To complement the bulk rheological characterization, MPT was employed to directly evaluate how gel composition influences NP mobility within different hydrogels and SVB. Specifically, the mobility of PP and PP HA NPs was assessed in P3 0.1%, P3 1%, and P4 0.1% hydrogels, as well as in SVB. The goal was to investigate how variations

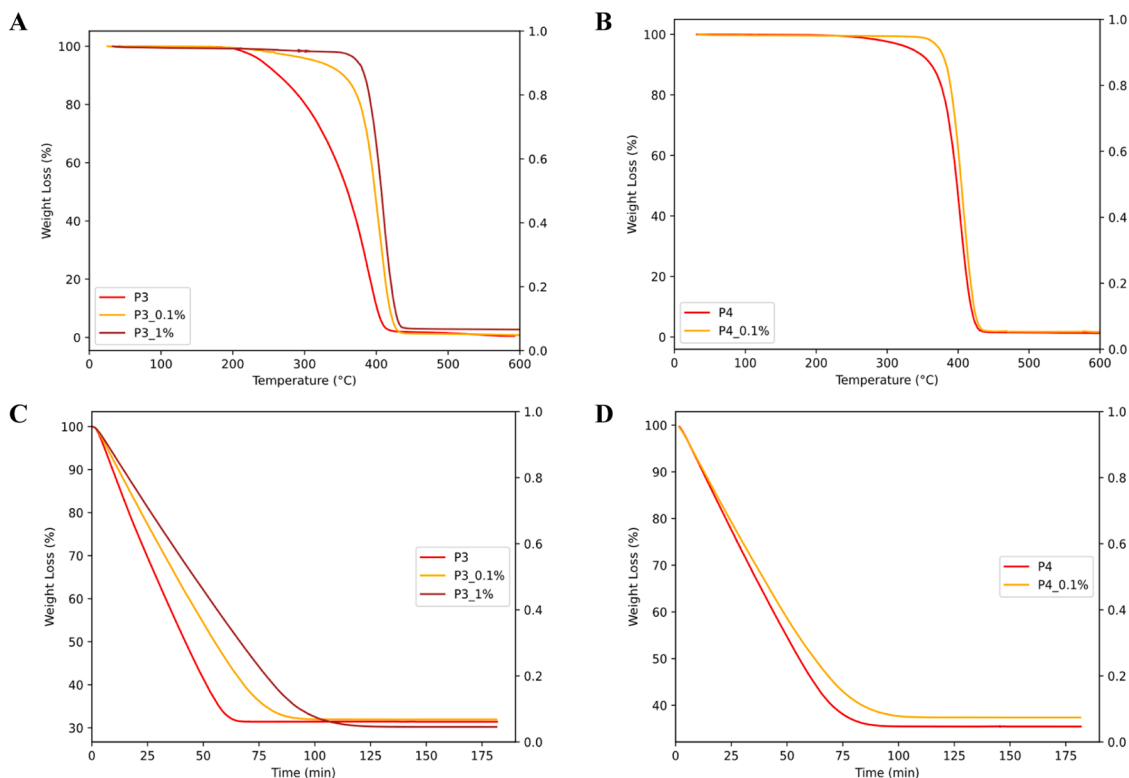


Fig. 3. TGA curves relating to lyophilized (A, B) and hydrated (C, D) gels.

Table 3

Start temperature, end temperature, DTG start, DTG peak, DTG end of the dynamic TGA analyses of lyophilized hydrogel.

Sample	Start (°C)	End (°C)	DTG start (°C)	DTG peak (°C)	DTG end (°C)
P3	181.1 ± 14.6	450.2 ± 11.1	151.5 ± 3.9	388.8 ± 3.1	447.9 ± 8.9
P3 0.1%	201.9 ± 10.5	438.7 ± 8.8	282.5 ± 21.4	400.0 ± 2.5	421.0 ± 2.3
P3 1.0%	243.4 ± 22.4	456.9 ± 9.5	322.5 ± 34.0	383.2 ± 5.6	464.1 ± 19.2
P4	249.8 ± 0.4	455.4 ± 18.3	260.6 ± 24.6	401.3 ± 6.4	461.1 ± 11.9
P4 0.1%	326.8 ± 15.6	474.8 ± 7.2	302.0 ± 26.5	402.8 ± 2.4	477.1 ± 3.6

Table 4

End time and weight loss of the hydrated hydrogel after isothermal TGA analyses.

Sample	End time (min)	Weight loss (%)
P3	78.8 ± 8.5	69.8 ± 0.8
P3 0.1%	94.1 ± 2.2	69.5 ± 1.1
P3 1%	122.8 ± 1.5	71.1 ± 1.0
P4	96.4 ± 0.3	67.9 ± 0.2
P4 0.1%	116.9 ± 8.2	63.0 ± 0.5

in poloxamer concentration, which shown to influence gel rheology, and HA content can be independently modulated to control and prolong NP diffusion and migration time toward the vitreous.

MSD analysis was performed, from which both the diffusion coefficients and the effective viscosity experienced by the NPs were calculated. NP mobility, expressed as MSD (Fig. 4A-B), was markedly higher in SVB at all explored times. At 4 s (3.8 s for SVB), MSD increased by approximately 6- to 40-fold for PP NPs and 4- to 40-fold for PP HA

NPs (Table S1).

Fig. 4E shows that both poloxamer and HA content significantly affected NP mobility. In particular, increasing poloxamer content from P3 0.1% to P4 0.1%, which corresponds to enhanced rheological properties, resulted in a reduction in mobility at 4 s of about 8-fold for both PP and PP HA NPs (Table S1). Comparing P3 0.1% and P3 1% hydrogels, whose rheological properties were not significantly altered by HA content, a marked decrease in mobility was nevertheless observed. Specifically, PP NP mobility in P3 1% decreased by about 7-fold, while PP HA NP mobility decreased by approximately 11-fold, reaching values slightly but significantly lower than those measured for PP HA NPs in P4 0.1%.

The mobility regimes, assessed through the MSD slope, were also evaluated. As shown in Figs. 4A-B, NP motion was sub-diffusive in all conditions, irrespective of HA presence on NPs. The MSD slope of PP NPs decreased to 0.87 and 0.85 in P3 1% and P4 0.1%, respectively, while PP HA NPs exhibited an even stronger reduction, reaching their lowest values, 0.82, when both the hydrogel and the NPs contained HA (P3 1% + PP HA NPs).

Considering the different sizes of PP and PP HA NPs used for the characterization of gels and SVB (153 nm for NP PP, 231 nm for NP PP HA), which intrinsically affect particle mobility (see eqs. 5 and 6), the time-dependent creep compliance $J(t)$ was also calculated. This mechanical parameter is independent of NP size and provides insights into the elastic ($J(t)$ constant), viscous ($J(t)$ linearly increasing with time), or viscoelastic character of the material ($J(t)$ power-law function of time with an exponent between 0 and 1). The results obtained are shown in Figs. 4C-D illustrating the creep trend, while Fig. 4F reports $J(t)$ values at 4 s.

Finally, gel viscosity was evaluated. As shown in Fig. 4G, SVB displayed the lowest viscosity, with reductions ranging from 6- to 35-fold compared to formulated hydrogels when measured with PP NPs, and from 4- to 40-fold when measured with PP HA NPs (Table S3).

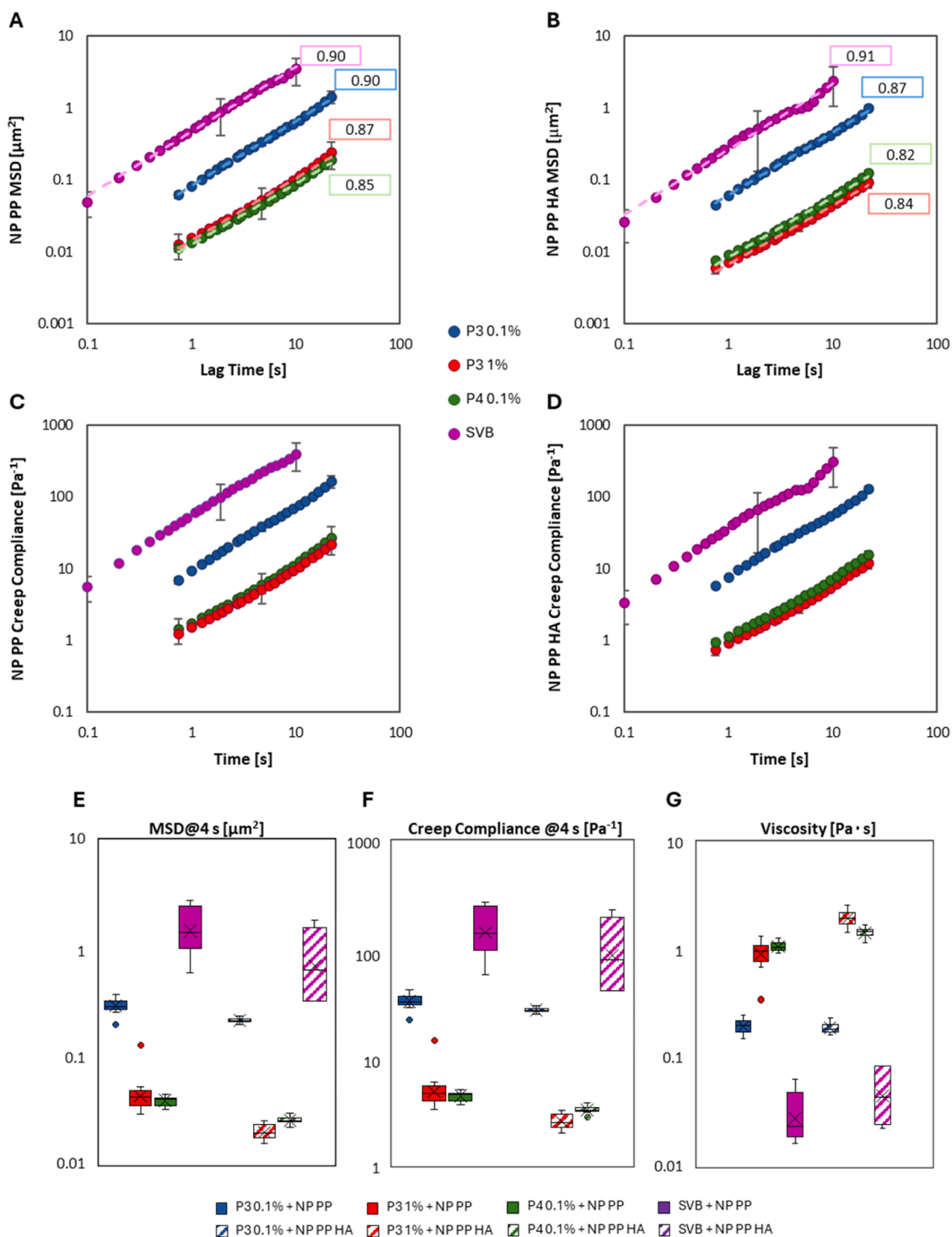


Fig. 4. Ensemble-averaged MSDs (A, B) and corresponding creep compliance (C, D) measured with PP (A, C) and PP HA (B, D) in SVB (violet), P3 0.1% (blue), P3 1% (red), and P4 0.1% (green). Box plots (mean, median, interquartile range, and outliers) of MSDs (E) and creep compliance (F) at 3.8 s for SVB (violet) and at 4 s for P3 0.1% (blue), P3 1% (red), and P4 0.1% (green), as well as viscosity values (G) measured with PP (solid boxes) and PP HA (hatched boxes).

3.7. In vitro studies

3.7.1. Determination of the cytotoxicity

Biocompatibility was evaluated using a retinal pigment epithelial cell line (ARPE-19). Cell viability was determined using the MTT assay, which measures intracellular metabolic activity and provides an indirect indication of cell viability (Fig. 5A and B). According to EN ISO 10993-5:2009 standards, materials achieving a relative cell viability $\geq 70\%$ in

the MTT assay are considered non-cytotoxic. Thus, the results showed that none of the hydrogels exhibited cytotoxic effects at the tested concentrations. An analog experiment was performed to evaluate NP biocompatibility. ARPE-19 cells were exposed to a concentration range of both B-PP and B-PP-HA NPs, for 24 and 48 hours (Fig. 5C and D) and cell viability was evaluated by MTT assay. The results demonstrated absence of cytotoxicity under all tested conditions.

Moreover, to exclude potential cytotoxicity due to a synergistic effect

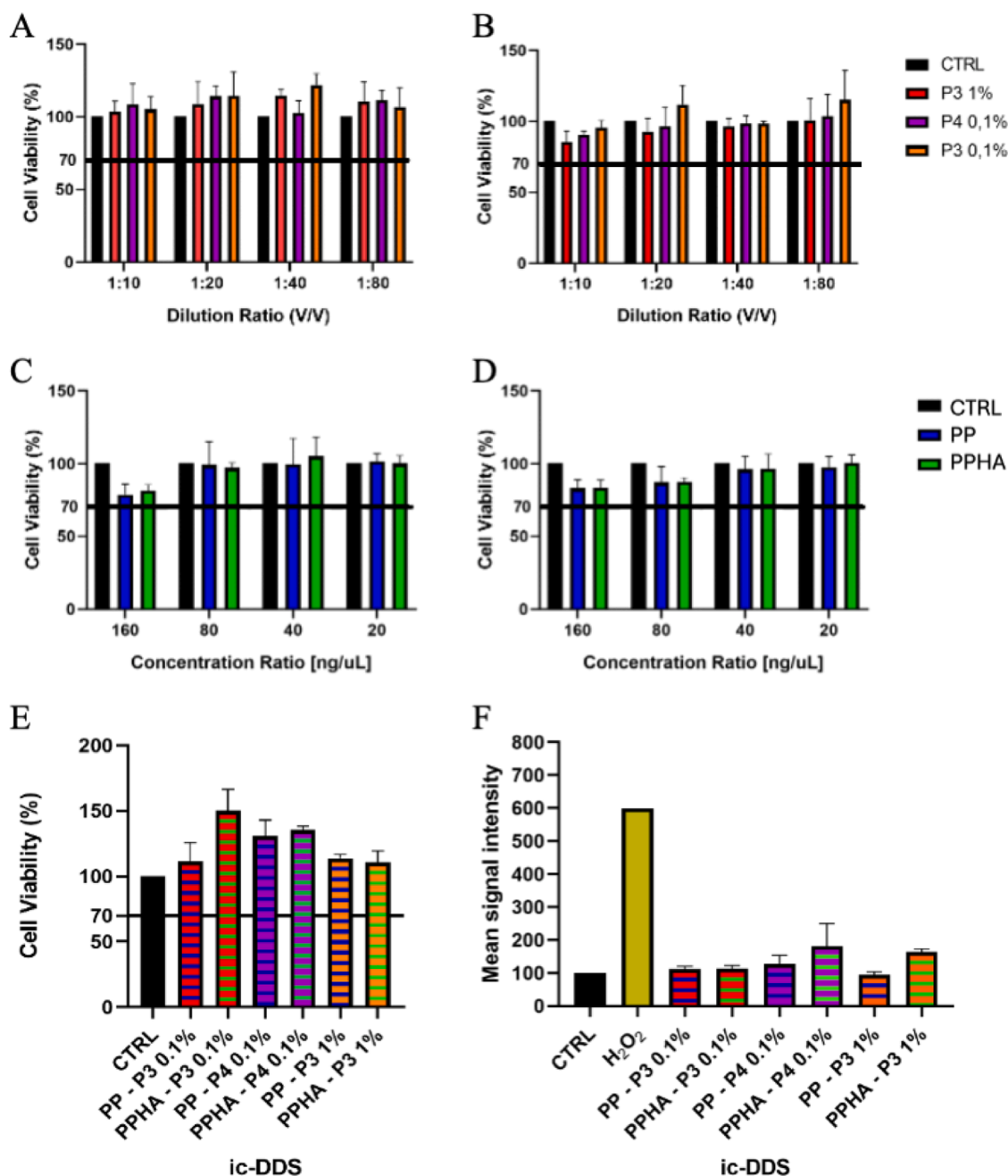


Fig. 5. Measurement of cell cytotoxicity by MTT assay showing viability of ARPE 19 cells exposed to: (A) a dose-response curve of P3 1%, P4 0.1%, and P3 0.1% hydrogels at 24 h and (B) 48 h; (C) dose-response curve of PP and PP HA B-NPs at 24 h and (D) 48 h; (E) 48 h of treatment with different ic-DDS (E). The ISO threshold for MTT cytotoxicity, a reduction of cell viability below 70% compared to control, is shown by the black line. (F) Intracellular ROS measure after treatment with different ic-DDS. Cells treated with H₂O₂ were used as control. Data are presented as the mean $\pm$ SD compared to untreated cells, from at least three independent experiments ($n = 3$).

between the hydrogels and NPs, cells were incubated for 48 h with cDDS formulated at the highest concentrations of the two components (Fig. 5E). None of the tested cDDS influenced cell viability, confirming the absence of synergistic cytotoxic effect between the hydrogels and NPs.

Furthermore, to determine whether the cDDS formulations could induce oxidative stress, ARPE-19 cells were incubated with the different cDDS formulations described above. After 48 h, intracellular reactive oxygen species (ROS) production was assessed using the CellROX® Green Oxidative Stress Reagent (Fig. 5F). The results demonstrated that none of the evaluated cDDS formulations induced significant intracellular ROS accumulation.

3.7.2. Cellular uptake of NPs

Fluorescence microscopy images (Fig. 6) show cell nuclei stained in blue (DAPI) and NPs in red (PLGA RHOD), confirming their successful internalization. After 24 hours, both PP and PP HA NPs were predominantly localized in the cytoplasm.

4. Discussions

In this study, we investigated the effect of HA in modulating the physicochemical properties of cDDSs composed of a thermosensitive poloxamers/HA hydrogel embedding PLGA-based NPs, with a without HA surface decoration. The work aimed to assess how HA affects

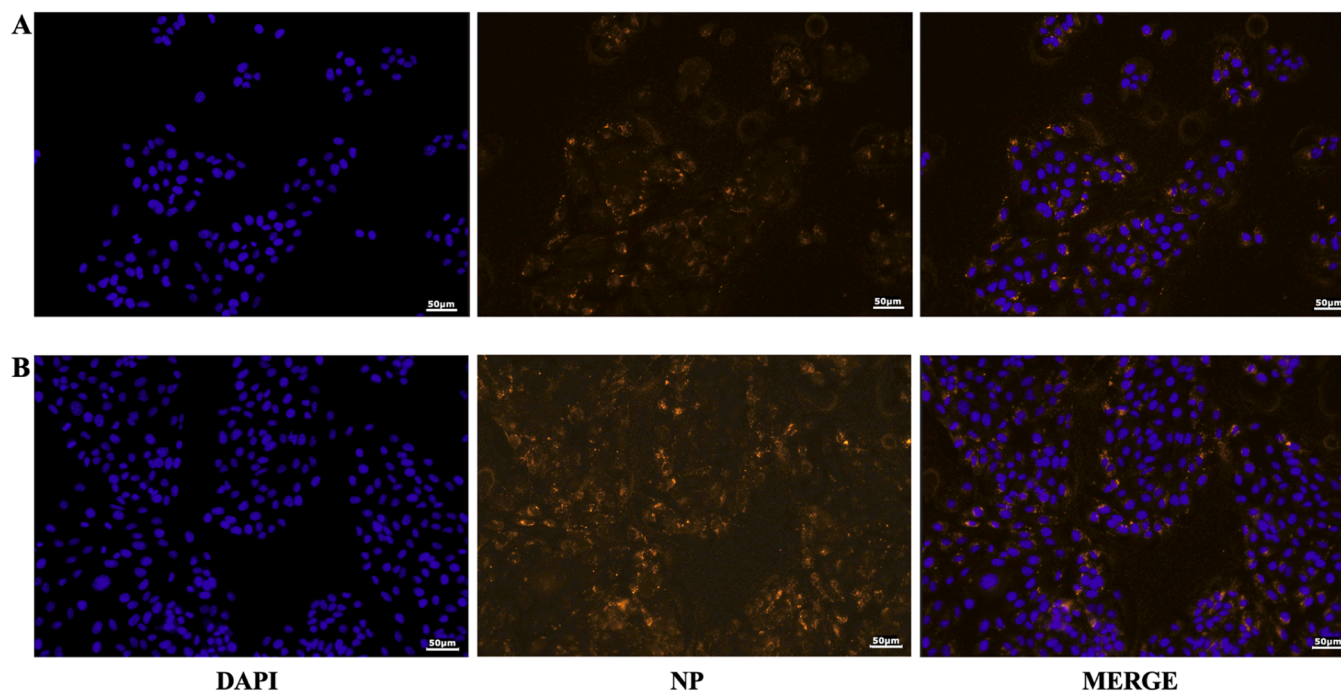


Fig. 6. Fluorescence images of ARPE cells incubated with fluorescent PP (A) or PP HA (B) (20 ng/ μ l) for 24 h at 20X. Bar scale 50 μ m.

hydrogel properties and NP transport within the gel matrix and in SVB.

Hydrogels were first characterized in terms of storage stability, sterilization, and injectability, which are pivotal needs for intravitreal formulations. In this context, the preservation of rheological properties after sterilization is also central since sterility is mandatory for ocular dosage forms. Several sterilization methods are available, including physical, thermal, and chemical approaches. However, terminal sterilization by autoclaving after vial sealing is generally preferred when it is compatible with the formulation. All hydrogels maintained their rheological features during storage and after autoclave sterilization, thus confirming the applicability in pharmaceutically relevant processing conditions. The rheological behavior was also maintained after the injection through fine gauge needle 27G used in the clinical setting for intravitreal injection to minimize tissue damage and ensure precise delivery (Melo et al., 2021), showing that the formulations can withstand the shear stress associated with injection. Regarding injectability, it must be underlined that injection force values should be interpreted as comparative estimates rather than direct measurements, since injection force in the clinics may depend on syringe geometry, needle gauge and length, injection rate, injection volume, temperature during handling, and operator-dependent factors (Lee et al., 2022). Injection force values increased with polymer concentration. Compared with P3 0.1, P3 1 showed a roughly 2.5-fold higher injection force, indicating the strong effect of HA content on injectability, even at < 1% concentrations. At the same HA concentration, P4 0.1 required an approximately 5-fold higher force than P3 0.1, highlighting the contribution of poloxamer content.

TGA analyses of lyophilized and hydrated samples revealed a clear effect of HA content. In lyophilized gels, the presence of HA shifted the onset of thermal degradation to higher temperatures, indicating an enhanced thermal stability. Similarly, in hydrated samples, HA delayed the evaporation process, with a more pronounced effect at increasing HA concentrations. This behavior can reasonably be attributed to the strong affinity of HA for water. In aqueous environments, HA forms highly swollen random coils, and its macromolecular chains preferentially interact with water molecules rather than with other segments of the same or neighboring macromolecules. This phenomenon has been extensively documented in the literature (Fouissac et al., 2002); (Mendichi et al., 2003); (Mayol et al., 2010); (Kutálková et al., 2020),

(Taweechat et al., 2020).

In situ-forming injectable implants, based on thermosensitive hydrogels for intravitreal drug delivery, has been gaining increasing attention within the scientific community. In this context, in vivo results are also very promising (Annala et al., 2025; Jiang et al., 2025; Li et al., 2025). In particular, the injection of small volumes has been shown to cause no adverse effects and full vitreal clearance of the hydrogels. More in detail, since the vitreous chamber is a closed system with a volume of approximately 4–5 mL, larger injected volumes may induce transient intraocular pressure (IOP) spikes, which have been associated with repeated intravitreal administrations. Consequently, the injected volume is typically limited to 50–100 μ L, depending on the drug formulation. Although IOP generally returns to normal within 30 minutes after injection, higher pressure increases have been reported in the absence of reflux and when using small-bore needles (Allmendinger et al., 2021).

Despite these encouraging results, drug release from hydrogels remains difficult to control, as swelling, diffusion, and degradation-driven mechanisms often result in a pronounced burst release (Jiang et al., 2025; Karimi et al., 2024; Xie et al., 2015a). Accordingly, several ocular cDDSs based on NP-loaded hydrogels have been proposed for intravitreal delivery, showing improved control on drug release and reduced burst effects (Ottonelli et al., 2022) (Zhang et al., 2021) (Wen et al., 2025) (Sapino et al., 2019) (du Toit et al., 2021). Moreover, when NPs are smaller than 400 nm, as in the present study, transparent composite hydrogels can be obtained, which is particularly relevant for intravitreal applications and may help minimize visual disturbance after administration (Fig. S1, S2, S3 in supplementary data).

In this context, NP diffusion within both the gel matrix and in SVB to be a crucial design parameter, we sought to investigate the role of HA in modulating this aspect.

The SVB employed in this study corresponds to a low-viscosity HA–agar vitreous mimetic formulation previously validated as in vitro reference model for assessing NP diffusion behavior under vitreous-like viscoelastic conditions (Lorenzo Lopez et al., 2024). Nevertheless, this simplified in vitro model does not aim to fully reproduce the biological heterogeneity of the human vitreous body, including age-dependent liquefaction, pathological remodeling, or patient-specific variability, but rather to provide a controlled and reproducible vitreous-like

environment in which nanoparticle transport could be directly compared by MPT. Hydrogel composition significantly influenced NP mobility. Comparing NP mobility in P3 0.1% and P4 0.1%, it was evident that increasing poloxamer content led to a reduction in mobility. A similar trend was observed comparing NP mobility in P3 0.1% and P3 1%. In this case, the reduced mobility in P3 1% was associated with the increased HA content, which exerted an even stronger effect than poloxamer (Table S1). Overall, these findings indicate that HA reduces NP diffusion, regardless of whether the NPs are functionalized with HA. Notably, this hindrance was further accentuated when HA is present on both the NP surface (PP HA) and in higher concentration within the hydrogel matrix (P3 1%). The use of HA–agar vitreous mimetic systems as reference media for NP diffusion studies is also consistent with previous reports (Lorenzo Lopez et al., 2024). Importantly, the MSD curves did not exhibit a plateau within the investigated timescale, which would be expected in the case of complete nanoparticle immobilization or trapping within the hydrogel network. Instead, the observed sub-diffusive behavior indicates that nanoparticle transport is hindered but not completely suppressed within the hydrogel matrix. However, longer-term release studies will be required to define the *in vivo* transport timeframe.

These findings were confirmed both by the analysis of the mobility regimes through the MSD slope and by the trend of creep compliance. The MSD slope (Fig. 4A–B) decreased with increasing HA concentration (P3 1%) or poloxamer content (P4 0.1%) and the hindrance effect was further exacerbated when NPs were also HA-functionalized. Since MSD values are intrinsically influenced by NP size, the additional analysis of creep compliance and viscosity was used to complement raw mobility data and to interpret NP transport in terms of particle properties and local matrix mechanics.

Regarding creep compliance analysis, $J(t)$ values at 4 s were consistently higher when measured with PP NPs compared to PP HA NPs across all gel formulations and, to a lesser extent, in SVB (Fig. 4F, Table S2). This difference was particularly evident in the presence of higher HA content (P3 1%), highlighting the combined contribution of matrix composition and NP functionalization.

Gel viscosity further confirmed the above observations. These results corroborate the combined role of gel network properties, NP size and surface characteristics, and HA-mediated modulation of the local transport environment. In agreement with bulk rheology, microrheology confirmed that increasing poloxamer concentration strengthens the network and reduces NP mobility, while only HA-rich formulations revealed an additional hindrance, reflecting local matrix–particle interactions not captured by bulk measurements. The more negative zeta potential of HA-coated MPs indicates the exposure of polyanionic HA at the NP surface, protruding outwards. This may enhance HA interactions with both hydrogels and SVB, which contains the same polysaccharide, likely through HA–HA entanglements.

As previously discussed, a critical requirement for the thermosensitive gel is its ability to initially retain NPs to minimize the risk of visual impairments, while allowing their subsequent release and free diffusion in the vitreous, enabling them to reach target cells, be internalized, and effectively deliver the drug. To evaluate this capability, another aspect investigated in the present study was the internalization of NPs by ARPE-19 cells, to evaluate whether, once released from the polymeric gel matrix and diffused into the vitreous body, the NPs could be effectively taken up by target cells. The results confirmed that both types of NPs were successfully internalized by ARPE-19 cells, predominantly localizing within the cytoplasm. Finally, cytotoxicity assessments demonstrated that all hydrogel platforms, NPs and cDDS exhibited good biocompatibility at the tested concentrations. Indeed, according to the European standard PN-EN ISO 10993-5:2009, a material is considered non-cytotoxic if it maintains a relative cell viability of $\geq 70\%$ compared to the control, as measured by the MTT assay. *In vitro* experiments validated the biocompatibility of the analyzed hydrogels and NPs, that didn't induce cell mortality. Moreover, the analysis of the different

cDDSs, at highest concentrations of the two components, confirmed that there was no synergistic cytotoxic effect. Furthermore, the biocompatibility of the cDDSs was supported by a lack of significant intracellular ROS production in ARPE-19 cells following treatment.

5. Conclusions

This study provides important evidence supporting the feasibility of an injectable cDDS based on NPs embedded within a thermosensitive hydrogel. Overall, the developed platform demonstrated favorable physicochemical and biological characteristics, maintaining its structural and functional properties throughout the manufacturing process and under conditions relevant to clinical administration. The combination of a poloxamer-based hydrogel with HA-functionalized NPs enabled the formation of a biocompatible system capable of gelling under physiological conditions and modulating NP mobility within the matrix. The reduced diffusion of NPs observed within the hydrogels suggests the potential to prolong their residence time following intravitreal administration, while their efficient uptake by ARPE-19 cells indicates the ability of the system to promote intracellular delivery.

Taken together, these findings highlight the potential of the proposed cDDS as a platform for sustained drug delivery to the posterior segment of the eye. Future studies will focus on incorporating a suitable therapeutic agent into the NPs and further optimizing the formulation to achieve controlled drug release over extended periods while minimizing the initial burst effect.

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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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.carpta.2026.101189](https://doi.org/10.1016/j.carpta.2026.101189).

Data availability

Data will be made available on request.

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