

A rationally designed polypeptide-based hybrid platform for targeted intranasal brain drug delivery

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ABSTRACT

Intranasal administration represents a safe and non-invasive route for drug delivery to the brain; however, clinical translation remains limited due to anatomical and physiological barriers. We present a modular hybrid biomaterial platform (NanoInBrain) that bypasses the blood-brain barrier via the olfactory route and enables central nervous system (CNS) drug delivery. The platform integrates a rationally designed polypeptide-based nanocarrier with a depot-forming hydrogel vehicle - a hyaluronic acid-poly-L-glutamate crosspolymer (HA-CP, Yalic®) - adapted from dermatological applications to enhance nasal mucosal retention and brain uptake. We engineered the nanocarrier system using star-shaped poly-L-glutamate (StPGA) architectures and systematically tuned physicochemical properties to optimize mucosal interaction and CNS diffusion. We introduced mucoadhesive and mucodiffusive functionalities via C-terminal odorranalectin (OL) conjugation, which improved nasal epithelium permeation through receptor-mediated mechanisms. Redox-responsive disulfide crosslinking (StPGA-CL-SS) further enhanced mucosal transport by enabling thiol-mediated anchoring to mucin glycoproteins, outperforming inert click-crosslinked variants. *Ex vivo* Franz diffusion studies and a nasal-mucosa-on-chip model demonstrated robust permeation, with *in vivo* imaging confirming brain distribution and intracellular uptake in neurons and microglia. Incorporation of HA-CP prolonged nasal residence (~4 h) and increased total brain accumulation while being well-tolerated. This new platform combines architectural tunability, bioresponsive surface chemistry, and depot-mediated delivery in a scalable, biocompatible nose-to-brain delivery system with potential for treating neurological disorders.

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1. Introduction

Neurological disorders - an escalating global health crisis - account for more than 15 % of global health loss, surpassing both cancer and cardiovascular disease in combined impact [1,2]. The current lack of effective treatments underscores the need for innovative therapeutic strategies. Intranasal administration has emerged as a promising route for the delivery of therapeutics to the central nervous system (CNS) as it avoids the blood-brain barrier (leveraging direct olfactory/trigeminal pathways for rapid CNS access), circumvents hepatic first-pass metabolism and gastrointestinal degradation [3–5], offers enhanced patient compliance, and supports significantly greater brain bioavailability of drugs compared to intravenous delivery [6,7]. Unfortunately, challenges associated with intranasal administration (e.g., rapid mucociliary clearance, enzymatic degradation of the therapeutic agent, and poor epithelial permeability for hydrophilic drugs) have limited the efficacy of associated therapeutics.

Biomaterials science has emerged as a central discipline in overcoming translational bottlenecks by enabling the design of nanocarriers with tunable architectures, biodegradability, and functional versatility. Nanoformulations based on lipids or polymers can mitigate specific barriers by enhancing mucosal adhesion, protecting payloads, and allowing controlled release [8]; however, only a few have progressed to clinical evaluation [5,7,8]. Major obstacles to the clinical translation of intranasal delivery include anatomical constraints in humans, poor drug deposition in the olfactory region, rapid mucociliary clearance, physiological variability, and the complex design requirements for nanocarriers (balancing retention with tissue penetration and subsequent brain diffusion). Challenges related to the need for scalable manufacturing and the lack of reliable preclinical models and insufficient long-term safety data also hinder regulatory approval and clinical translation [9–11].

Polypeptide-based drug nanocarriers [12] offer distinct advantages through precise molecular customization, reproducible synthesis, and predictable biological performance; furthermore, they display inherent biocompatibility and degrade into natural amino acids, reducing toxicity concerns. Concerning intranasal drug delivery to the brain, elastin-like polypeptides display significant CNS accumulation after intranasal administration [13] while additional strategies using polypeptide-based hydrogels, nanostructures, or self-assembled systems have supported enhanced mucosal retention while protecting sensitive peptides or protein drugs, often achieving improved brain delivery compared to systemic administration routes [14]. In contrast, lipid-based nanocarriers (including liposomes, solid lipid nanoparticles, and lipid-gelatin hybrids) remain among the most widely explored systems for intranasal delivery [15,16]. Their advantages include efficient drug encapsulation and ease of scale-up; however, they often face limitations such as rapid disassembly, limited functionalization capabilities, and the need for post-synthetic surface modifications (e.g., modification with polyethylene glycol [PEG; PEGylation] or surfactants) to enhance mucoadhesion or targeting. These modifications introduce variability and reduce design precision [17]; therefore, polypeptide-based systems offer advantages for intranasal administration when comparing classes, as they support molecular-level engineering and site-specific ligand conjugation, enabling structurally defined, multifunctional drug nanocarriers with bioresponsive properties [12,18]. A body of evidence now highlights the influence of nanoplatform size and architecture on biological fate, including altered cellular internalization efficiency, systemic circulation kinetics, and organ-specific biodistribution patterns following intravenous delivery [19–21]. Poly-L-glutamate (PGA) provides functionalization capacity and architectural versatility, allowing the formation of nanostructures such as linear, star-shaped, or crosslinked assemblies [18,22]. PGA's versatility also allows the design of tailored architectures that support optimized biological interactions [6,7,12]. The modification of PGA with bioactive and mucoadhesive moieties enables the fine-tuning of

physicochemical and biological properties for targeted drug delivery applications while the ability to self-assemble into nanosized structures [18] and accommodate multivalent functionalization make PGA a robust platform for engineering responsive and multifunctional nanocarriers.

Physicochemical properties (i.e., density, size, shape, and hygroscopicity) significantly influence the deposition and transport of intranasally administered nanocarriers through the nasal cavity [23] and their interaction with the extracellular brain environment [24–26]. Although the mucus layer displays a negative charge, PGA's polyanionic nature does not preclude effective mucosal interaction. Beyond electrostatic considerations, PGA engages in hydrogen bonding and physical entanglement with mucin glycoproteins, facilitating mucoadhesion. Notably, negatively charged polypeptides such as PGA can enhance permeability by reducing unfavorable interactions with mucus and, as such, have been explored as tumor vaccine adjuvants, antigen delivery systems, and intranasal vaccination platforms [27].

In formulations incorporating thiol groups and disulfide linkages, covalent disulfide exchange provides additional mucoadhesive anchoring independent of charge; these non-electrostatic mechanisms support stable mucus association and are consistent with behavior reported for other polyanionic and zwitterionic mucoadhesive polymers [28,29]. Moreover, PGA's hydrodynamic and structural properties influence the extent of brain tissue diffusion. These design-dependent features determine the success of mucosal crossing and the eventual biodistribution and therapeutic reach within the CNS. Thus, leveraging PGA's multivalency and adaptability offers a rational pathway to develop scalable and targeted intranasal drug delivery systems for neurological disorders.

This study focuses on the rational design and development of an intranasally administered nanoplatform (NanoInBrain) through the systematic evaluation of: (i) PGA architectures to fine-tune physicochemical properties and enhance mucosal transport; (ii) stabilizing self-assembled nanocarriers via crosslinking to alter important parameters such as diffusion [18,30–32] and provide mucoadhesive properties [33]; (iii) incorporating functional ligands (odorranalectin [OL], hyaluronic acid [HA], and didocosahexaenoic acid [DHA]) to improve mucosal interaction, retention, and CNS uptake; and (iv) vehicle formulation to enhance mucosal retention or function as a depot system to sustain/-maximize drug concentrations in the brain. We employed disulfide bonds to provide stability via biodegradable crosslinking of StPGA nanostructures (StPGA-CL-SS) while conferring redox sensitivity [34]. Thiolated polymers derivatized with sulfhydryl groups on their polymeric backbone can form disulfide bridges with cysteine-containing glycoproteins of the mucosa [35–37]; consequently, this interaction supports additional mucoadhesive properties, thereby prolonging residence time and facilitating mucodiffusion [33].

In the design of nose-to-brain drug delivery systems, ligand selection remains critical to overcoming anatomical and physiological barriers such as limited residence time, mucociliary clearance, and poor targeting specificity. We screened three rationally selected functional moieties – OL [38–40], HA [41,42], and DHA [43] – to address complementary challenges to intranasal CNS delivery. OL – a small, cyclic lectin-like peptide – specifically binds to L-fucose residues on the olfactory epithelium, promoting preferential deposition in the olfactory region and enhancing brain targeting [40]. Low immunogenicity, chemical stability, and efficient conjugation make OL a practical alternative to larger ligands such as transferrin or monoclonal antibodies, which often face translational hurdles related to receptor heterogeneity, manufacturing complexity, and immunogenic risk [10,44]. We selected HA as a mucoadhesive and biocompatible polymer with extensive clinical precedent [42,45]; unlike chitosan derivatives (which can exhibit variability in mucoadhesion and mucosal irritation), HA offers a well-characterized safety and biodegradability profile and interacts with CD44, which provides relevance to neuroinflammation and glioma [46]. We incorporated DHA – an omega-3 fatty acid abundant in neuronal

membranes – to support mucosal permeation and enhance lipid-mediated brain uptake [47]. Beyond delivery-enhancing properties, DHA provides intrinsic neuroprotective and anti-inflammatory effects [48], offering a safer and biologically relevant alternative to synthetic permeation enhancers or surfactants [44]. The selection of OL, HA, and DHA reflects a balance of specificity, safety, scalability, and functional performance. The use of OL (selective olfactory binding), HA (mucoadhesion and biocompatibility), and DHA (lipid-enhanced permeation and neuroaffinity) enabled the rational design of multifunctional nanocarriers addressing significant barriers in nose-to-brain drug delivery: mucosal retention, epithelial penetration, and brain targeting. By conjugating each ligand individually to PGA, we explored their impact on nasal permeation and brain uptake, identifying the most effective modification or combination thereof. This strategy allowed us to determine which ligand most effectively enhances PGA's potential as a brain-targeted delivery platform for future therapeutic applications. By tuning nanocarrier size, charge, architecture, and surface functionality, we aimed to engineer scalable, biocompatible, and translationally relevant nanocarriers for non-invasive, targeted CNS drug delivery.

Importantly, the development and clinical translation of intranasal therapeutics face a significant obstacle: the lack of appropriate models for rapid screening and evaluation. This status underscores interest in developing *in vitro* models that effectively replicate *in vivo* conditions driven by economic and ecological considerations to accelerate drug development and reduce animal use [23,49,50]. Establishing *in vitro-in vivo* correlations (IVIVCs) remains crucial for predicting *in vivo* drug deposition from nasally inhaled drugs based on *in vitro* measurements [51]. Current approaches rely on *ex vivo* models such as Franz diffusion cells with excised animal nasal mucosa [23,52] and *in vitro* systems using artificial membranes or cell lines, which, while cost-effective and standardized, fail to fully replicate *in vivo* conditions, particularly in predicting deposition site effects or residence time [49–51,53]. Vertical Franz cells with ovine nasal mucosa can provide insights into permeability and mucosal integrity [52], but do not account for dynamic factors such as mucociliary clearance or regional deposition. Despite these advantages, *ex vivo* models cannot reveal how formulation residence time or deposition site affects absorption and permeation, requiring *in vivo* confirmation to validate findings from the *ex vivo* model. These limitations mirror clinical observations where even potent drugs such as intranasal insulin or oxytocin achieve brain concentrations of <0.01 % of the administered dose, requiring formulations with absorption enhancers or mucoadhesive agents to improve retention and permeation [54–56]. Improving both *in vitro* and *in vivo* measurement methods that have emerged alongside organ-on-chip systems (still in early stages of development) [57] and *in-silico* modeling [50], combined with a deeper understanding of nasal deposition dynamics and the bio-nano interface, remains essential to improving IVIVC in the future.

Herein, our study reports that PGA-based nanocarrier architecture, size, and surface functionalization represent critical determinants of nasal mucosa permeation and effective brain delivery. In addition, incorporation into an HA-PGA cross-polymer (HA-CP, Yalic®) vehicle enhances intranasal transport and retention. Compared to widely studied intranasal delivery systems such as liposomes, chitosan nanoparticles, and lipid–gelatin hybrids, which often rely on post-synthetic modifications (e.g., PEGylation or surfactants) to enhance mucoadhesion or targeting but face challenges with rapid disassembly, mucosal irritation, and inconsistent penetration depth [15–17], our platform employs biodegradable disulfide crosslinking and intrinsic thiol-based mucoadhesive interactions [35–37], resulting in enhanced structural stability, residence time, and epithelial compatibility. Moreover, the decoupling of the nanocarrier core (StPGA) from the vehicle (HA-CP) allows for orthogonal optimization of each functional component, enabling a level of design precision and tunability rarely achieved in earlier polymeric or lipid-based systems [5,8,16]. These findings underscore the potential of this newly developed platform as a scalable, safe, and modular intranasal platform that overcomes the blood-brain

barrier and may enable the non-invasive treatment of neurological disorders.

2. Results and discussion

2.1. Design, development, and physicochemical characterization of a modular intranasal delivery platform

In this study, we developed and characterized a modular nanocarrier system designed to enhance intranasal delivery across the nasal epithelium and into the brain. As illustrated in Fig. 1, the platform centers on two components: an optimized PGA-based nanocarrier and a HA-based copolymer hydrogel (HA-CP).

Fig. 1A presents a representative star-shaped (St)PGA stabilized via biodegradable disulfide crosslinking (StPGA-CL-SS), with Fig. 1B reporting corresponding transmission electron microscopy (TEM) images and Fig. 1C confirming chemical modification via ¹H-NMR analysis. Fig. 1D–F report the characterization of HA-CP by SEC-RI (size exclusion chromatography using a refractive index detector) and fiber morphology by TEM. Fig. 1G provides a schematic description of the final hybrid assembly (StPGA-CL-SS/HA-CP), while Fig. 1H provides a visualization via TEM. Together, these elements provide features (physicochemical stability, redox-responsive disassembly, and tailored mucoadhesive/mucodiffusive behavior) that combat the poor permeability of high-molecular-weight (Mw), hydrophilic, and charged therapeutics.

2.1.1. Optimizing the PGA-based nanocarrier

To investigate how nanocarrier architecture and surface chemistry influence performance, we compared linear (LinPGA), StPGA, and crosslinked StPGA variants, incorporating distinct targeting or mucoadhesive moieties. This comparative approach enabled us to link specific structural features (e.g., disulfide crosslinking and ligand conjugation) with physicochemical properties, mucosal interactions, and barrier penetration.

Architectural tunability and biomimetic functionality represent crucial differentiating features of our platform; many intranasal nanocarriers rely on rigid cores or require extensive post-synthetic modifications such as PEGylation or surfactant stabilization. In contrast, our polypeptide-based carriers support direct, covalent ligand conjugation and biodegradable crosslinking [12,22,30,31], allowing reproducible synthesis with predictable biological behavior. Unlike chitosan derivatives linked to mucosal irritation or ciliary toxicity [46], or lipid-based systems prone to rapid disassembly and limited surface chemistry [17,47,58], our StPGA-CL-SS nanocarriers provide thiol-mediated disulfide exchange for stable mucoadhesive anchoring, which enables both prolonged retention and controlled redox-responsive disassembly, ensuring efficient permeability while preserving mucosal tissue integrity.

We covalently labeled our nanocarriers with Oregon green (OG) or Cyanine 5.5 (Cy5) for *in vitro* and *in vivo* tracking. Fig. S1 provides an overview of all synthesized PGA-based nanocarriers. Fig. 1A–Table 1, SI Section S1.2, and Figs. S1–S19 report synthetic schemes and physicochemical characterization details. Notably, surface modifications did not disrupt the core PGA structure (Figs. S2, S10, S14, and S19).

We systematically compared the physicochemical properties of LinPGA [59,60], StPGA [22], and two crosslinked StPGA variants: one using a non-biodegradable copper-click linker (StPGA-CL) [18] (Fig. S3) and another using a novel biodegradable disulfide-based linker (StPGA-CL-SS) (Fig. 1A, Fig. S4). StPGA self-assembles into ~100 nm nanostructures in low-ionic-strength aqueous media, following ordinary-to-extraordinary transition behavior [18] but disassembles rapidly under physiological conditions.

We utilized copper-catalyzed azide-alkyne cycloaddition to stabilize StPGA assemblies, resulting in StPGA-CL with increased size and a negative Z-potential (Fig. S3) [18]. This architecture improves

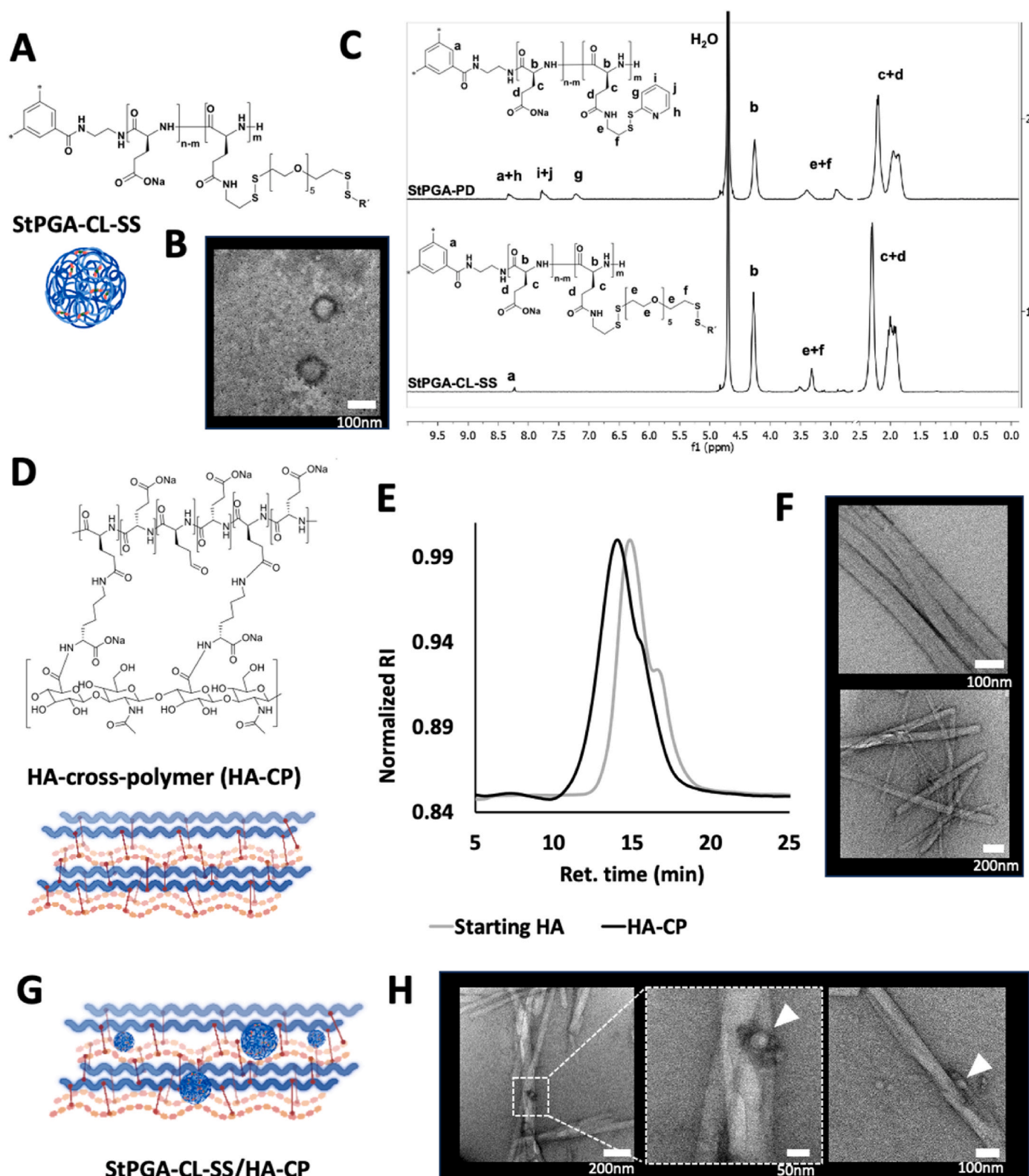


Fig. 1. Description of individual components and the final form of the modular intranasal delivery platform. (A) Chemical structure of StPGA-CL-SS and its schematic representation. (B) Representative TEM image of StPGA-CL-SS (1 mg/mL). Scale bar = 100 nm. (C) Comparison of ^1H -NMR spectra of StPGA-PD and StPGA-CL-SS in D_2O . (D) Chemical structure and schematic representation of HA-CP. (E) SEC-RI elugram for HA-CP and HA in aqueous media (100 mM NaNO_3 (pH 5); 5 mM PB). (F) Representative TEM image of HA-CP fibers (1.33 %w/v; 13.3 mg/mL). Scale bars = 100 nm (top) and 200 nm (bottom). (G) Schematic representation of StPGA-CL-SS/HA-CP. (H) TEM images of StPGA-CL-SS/HA-CP (1.33 % w/v). Scale bars (left to right) = 200, 50, and 100 nm. Part of the figure created using BioRender.com.

Table 1
Summary of physicochemical descriptors.

Compound name	Moiety Loading	OG loading (% wt) ^a	Size (Dh, nm ± SEM) ^b		PDI	Z-pot (mV)
			1 mg/mL	0.1 mg/mL		
LinPGA	n/a	2.1	9.5 ± 3.7	10.9 ± 2.3	0.287	−20.0 ± 2.2
StPGA	n/a	2.4	10.7 ± 3.5	13.0 ± 0.9	0.270	−25.1 ± 3.4
StPGA-CL	n/a	1.5	87.6 ± 4.4	n/a	0.277	−27.3 ± 0.4
StPGA-CL-SS	n/a	1.6	110.9 ± 3.4	n/a	0.267	−30.7 ± 0.2
Technique	AA/NMR					
StPGA-OL (C-term)	n.a./0.9	1.2	12.4 ± 1.5	9.7 ± 0.8	0.222	−20.2 ± 0.3.9
StPGA-OL (N-term)	3.8/3.4	2.2	9.1 ± 1.4	9.3 ± 1.2	0.253	−20.0 ± 2.2
Technique	¹H-NMR					
StPGA-HA80KDa	2:1 (ratio PGA:HA)	3.8	7.8 ± 0.4	9.3 ± 1.3	0.310	−25.5 ± 4.1
Technique	%mol, NMR					
LinPGA-DHA ₂	2.2	2.1	66.9 ± 8.2	n/a	0.245	−55.4 ± 1.2
LinPGA-DHA ₆	6.4	1.5	36.4 ± 5.0	n/a	0.273	−57.2 ± 1.6
StPGA-DHA ₂	1.5	1.9	30.3 ± 2.2	34.5 ± 8.3	0.298	−45.1 ± 3.5
StPGA-DHA ₆	6.0	0.9	35.1 ± 5.2	42.8 ± 4.5	0.277	−44.6 ± 4.7

^a Obtained by UV-Vis.

^b Obtained by DLS. % wt = weight percent loading; HA = hyaluronic acid; OG = Oregon Green; ¹H-NMR = Proton Nuclear Magnetic Resonance; CL = crosslinked via click chemistry; CL-SS = crosslinked via disulfide bonding; C-term = C-terminal; Dh = hydrodynamic diameter; DHA = didocosahexaenoic acid; n/a = not applicable; N-term = N-terminal; OL = odorranalectin; StPGA = star-shaped poly-L-glutamic acid.

lymphotropic properties behavior following intravenous administration, outperforming both LinPGA or non-crosslinked parental StPGA [18]. Moreover, StPGA-CL supports modular drug conjugation strategies for the rational tuning of release kinetics and payload ratios [30–32,61].

For intranasal delivery, large, charged, and hydrophilic molecules face limited permeability across the nasal epithelium [62,63]. To overcome this obstacle, we introduced mucoadhesive and redox-responsive features using disulfide (S-S) and thiol (–SH) chemistry [37]. The resulting StPGA-CL-SS platform (Fig. 1A and S2) exhibited redox-sensitive disassembly, enhanced biodegradability, and improved mucosal interaction, all of which support sustained mucosal residence and deeper tissue penetration.

The synthesis of StPGA-CL-SS began with the modification of StPGA using pyridyl disulfide (PD)-cysteamine, chosen for its high thiol–disulfide exchange efficiency and stability as a robust leaving group [64]. This modification enabled precise disulfide crosslinking and the formation of stable nanocarrier assemblies under mild, aqueous conditions. The PD moiety also allows for broad reactivity with thiol-containing compounds while preventing reverse exchange reactions, ensuring directional bond formation and long-term structural stability [64,65].

Initial attempts to assemble StPGA-PD at high concentration failed to yield stable nanostructures, unlike with the azide or propargyl unimers to achieve StPGA-CL [18], suggesting that PD's increased hydrophobicity disrupts aqueous self-assembly. We therefore explored various synthetic strategies to improve stability (Fig. S4A). Among these, the inverse nanoprecipitation protocol (Fig. S4B) demonstrated the highest efficiency for disulfide bond formation (Fig. S4C) [66,67], yielding stable nanoconstructs with sizes between 80 and 110 nm (hydrodynamic radius [Rh] measured by DLS; Table 1 and Fig. S4D).

2.1.2. Ligand conjugation enhances mucosal interaction and targeting capabilities

As an alternative to crosslinking, we evaluated StPGA functionalization with mucoadhesive and targeting ligands (OL, HA, and DHA) to improve mucosal interaction and intranasal delivery.

For OL conjugation, we first added a glycine residue to the N-terminal tyrosine of OL to facilitate coupling while preserving the carbohydrate-binding site and disulfide bond (Fig. S5). We developed two synthetic routes for covalent attachment to StPGA via amide bond formation: one targeting the N-terminal amine of OL (Fig. S6) and the other targeting the C-terminal carboxylic acid (Fig. S7). A complication with N-terminal conjugation arose due to an internal lysine (Lys5), which introduces a second amine that may participate in side reactions;

however, its close spatial proximity to OL's disulfide bridge partly limited the formation of undesired products. Using this method, we achieved 3.8 % mol OL loading, as quantified by amino acid analysis (StPGA-OL(N-term); Table 1).

To enhance selectivity, we pursued a C-terminal conjugation strategy using Boc protection and mild coupling. Wen et al. previously reported selective OL conjugation to PEG-poly(lactic-co-glycolic acid) nanoparticles [38], requiring disulfide bridge cleavage, acetamidomethyl protection, and a thiol–maleimide linkage; however, the harsh deprotection conditions (iodine/acetic acid, pH 4) caused precipitation of StPGA, precluding its use. Instead, we adapted a one-pot aqueous deprotection strategy described by Kobayashi et al. [68], which employs CuSO₄ and 1,2-aminothiol under aerobic conditions (pH 7.0) to remove acetamidomethyl groups without disrupting the disulfide bridge.

We protected the OL N-terminal and Lys5 amines using tert-butyloxycarbonyl (Boc) groups and then coupled the protected peptide to StPGA pre-functionalized with mono-protected diaminoethane via its C-terminal carboxylic acid. Following coupling, we removed Boc groups using TFA (trifluoroacetic acid). ¹H-NMR spectra of OL and OL(Boc)₂ (Figs. S8–S9) confirmed complete protection and successful deprotection. We observed a final loading of the StPGA-OL(C-term) conjugate of 0.9 % mol (Table 1), confirming the efficiency of the strategy and reproducibility of the conjugation approach.

Circular dichroism (CD) analysis of both StPGA-OL variants revealed the retention of the unmodified StPGA's predominant random coil conformation, with no significant structural alterations following OL conjugation or OG modification (Fig. S2 vs. Fig. S10; LinPGA and StPGA vs. StPGA-OL(N-term) and StPGA-OL(C-term)). In StPGA-OL(C-term), minor changes such as a shift in the minimum of the main peak and the disappearance of the 220.7 nm signal likely reflect the combined influence of the StPGA backbone and OL's secondary structure [69]. In contrast, StPGA-OL(N-term) exhibited side reactions involving OL's Lys5, disrupting its organized structure and promoting a transition to random coils.

We next evaluated HA incorporation into StPGA by either chemical conjugation (StPGA-HA80kDa via amide coupling) or physical mixing (StPGA/HA80kDa). For chemical conjugation, we first linked N-Boc-1,6-hexanediamine to StPGA and then deprotected Boc before subsequent HA (80 kDa) coupling (Fig. S11). Physicochemical analysis confirmed the presence of a distinct product with a final StPGA:HA ratio of 2:1 (Figs. S12 and S13). CD spectra of StPGA-HA80kDa revealed superimposed signals from both polymers, consistent with a predominant random coil conformation and indicating that HA conjugation did not alter the overall structure (Fig. S14).

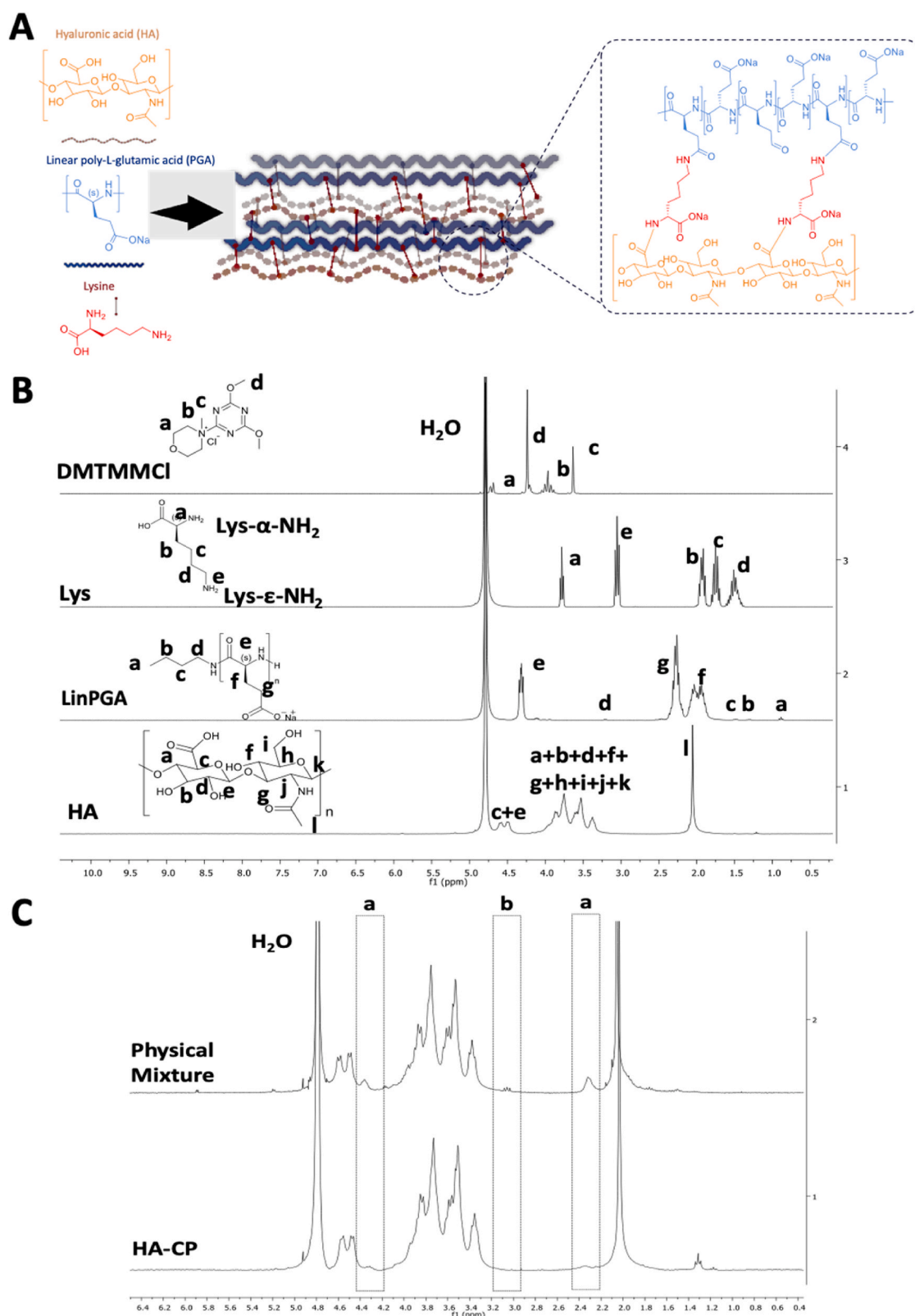


Fig. 2. Synthesis and characterization of HA-CP. (A) Chemical structures of individual components and HA-CP. (B) ¹H-NMR spectra in D₂O for starting materials with assigned protons. (C) ¹H-NMR spectra in D₂O for HA-CP and physical mixture showing PGA signals in HA-CP (a: Signal for LinPGA; b: Signals for Lys in HA-CP [not used for DOSY NMR studies due to low intensity]). (D) Schematic representation of DOSY-NMR array data treated according to the Stejskal-Tanner equation to compare the diffusion decay of HA, PGA, and Lys moieties. (E) Graphic representation of reduced viscosity at different concentrations of HA-CP or HA to reach the average Mw. (F) Representative TEM images for linear HA at 1w/v%. (left) and TEM images for HA-CP at 1w/v% (right). (G) Percentage of free amine determined by TNBSA assay in the crosslinked reaction media vs. alterations to pH. (H) Viscosity in cP (centipoise) for the crosslinking reaction media.

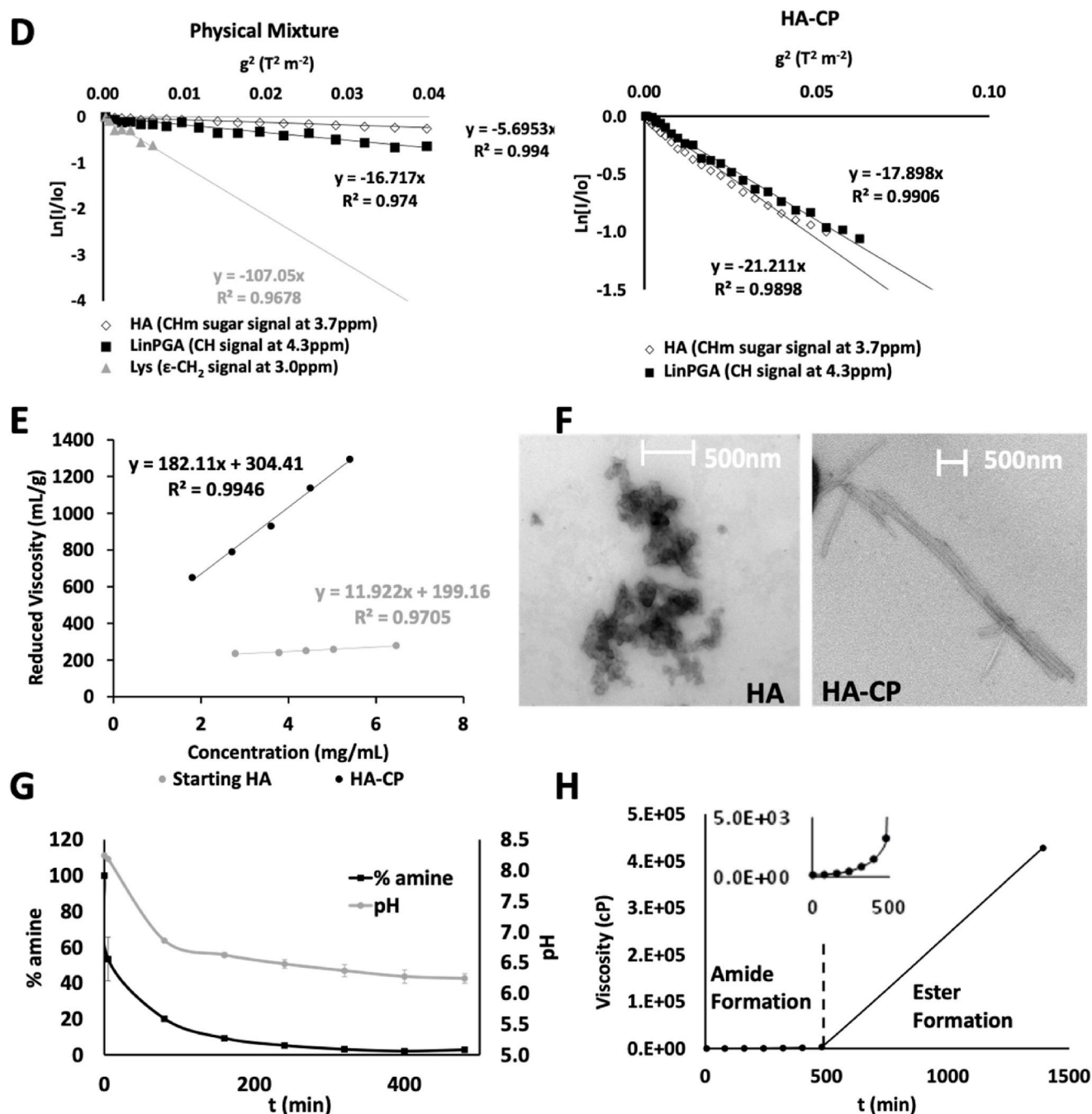


Fig. 2. (continued).

We then introduced DHA into StPGA and LinPGA via post-polymerization esterification using a reported protocol [49] (Fig. S15). We expected the combination of lipophilic DHA with hydrophilic PGA to increase nanostructure size and enhance mucosal penetration. To assess structural impact, we synthesized conjugates with 2 % and 6 % mol DHA loading (LinPGA-DHA2, StPGA-DHA2, LinPGA-DHA6, and StPGA-DHA6; Figs. S16–S19). As anticipated, DHA conjugation resulted in a 3–7-fold increase in Rh, compared to HA and OL derivatives (Rh, 10–13 nm; Table 1), consistent with the formation of nanosized, spherical aggregates previously reported by TEM [70]. Interestingly, higher DHA loading yielded smaller constructs, due to compaction from altered hydrophilic-lipophilic balance. We observed a less evident size reduction in StPGA, due to its more rigid, branched architecture [70]. CD spectra of PGA-DHA conjugates closely matched their unlabeled

counterparts (Fig. S19 vs. S2), confirming preserved random coil conformation and minimal structural perturbation.

Fig. S24 displays representative TEM and cryo-TEM images of StPGA, StPGA-OL, and StPGA-SS-CL with and without HA-CP, confirming the spherical morphology of StPGA and StPGA-SS-CL and demonstrating that StPGA-OL exhibits a more irregular structure. Notably, we observed elongated fibrous structures corresponding to HA-CP; furthermore, all three nanocarriers interacted with and surrounded HA-CP fibers. We performed imaging at a final concentration of 1 mg/mL, which allowed for visualization of particle morphology and spatial organization; these photomicrographs complement previous reports and support the structural characterization.

Together, these results demonstrate that the rational design of PGA-based nanocarriers - through architectural tuning, crosslinking

strategies, and functionalization with mucoadhesive or permeation-enhancing moieties - can modulate physicochemical properties, setting the stage for future structure-activity relationship studies using appropriate preclinical models to evaluate their potential in intranasal delivery.

2.1.3. Development of HA-CP as a vehicle to enhance nose-to-brain transport: synthesis, optimization, and physicochemical characterization

In addition to nanocarrier design, optimizing the delivery vehicle remains crucial to improving intranasal delivery. We hypothesized that HA-CP would support nose-to-brain transport due to its previously reported skin permeation-enhancing capabilities [71]. With its biocompatibility, mucoadhesiveness, and ability to influence nanocarrier mobility, we expected HA-CP to synergize with PGA-based systems by enhancing mucosal retention and permeation.

HA crosslinking can employ diverse methodologies [72]; here, we synthesized HA-CP via amidation by activating carboxylic groups on HA and LinPGA with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) and statistically crosslinking them using primary amines from Lys (Figs. 1D and 2A; Scheme S1). We optimized crosslinking efficiency by tuning pH, reagent stoichiometry, and the composition of HA, LinPGA, and Lys (Table S1). We monitored the reaction by $^1\text{H-NMR}$ and a 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay to quantify free amines. Optimal conditions (activation pH ~ 7 , coupling pH ~ 8 , 20 eq. DMTMM-Cl per Lys) yielded HA-CP with an Mw of ~ 150 kDa, more than triple the initial 50 kDa of HA (Fig. S21, Scheme S1, Table S1).

Analysis of the $^1\text{H-NMR}$ spectra confirmed the presence of HA and PGA in dialysis-purified HA-CP; we also identified Lys signals at a low-intensity ratio due to HA-CP composition ($\sim 1\%$ wt. Lys) (Fig. 2B and C). Rh values for HA and LinPGA experienced around a two-fold increase in the physical mixture when compared to HA-CP (Rh 10.4 nm vs. 17.9 nm), Diffusion-ordered spectroscopy (DOSY)-NMR analysis (Fig. 2D) revealed reduced diffusion in HA-CP compared to the physical mixture ($D = 1.1 \times 10^{-11} \text{ m}^2/\text{s}$ vs. $1.9 \times 10^{-11} \text{ m}^2/\text{s}$), confirming covalent crosslinking. Correspondingly, Rh values decreased from 17.9 nm to 10.4 nm in the physical mixture, supporting moderate crosslinking due to the limited Lys content (Table 2, Fig. S21). SEC further validated crosslinking, with HA-CP exhibiting a lower retention time and threefold Mw increase (145 vs. 49 kDa) (Table 2; Fig. 1E) [73]. Intrinsic viscosity, determined using the Mark-Houwink-Sakurada equation (S1.1.3) [74], reflected enhanced molecular size and chain entanglement consistent with a crosslinked polymer network (Fig. 2E). These results matched the SEC and DOSY findings (Table 2). TEM analysis revealed distinct morphologies: HA formed disordered aggregates, while HA-CP displayed elongated fibrillar structures, consistent with supramolecular hydrogel-like assembly (Figs. 1F and 2F), as expected from a crosslinked polymeric system.

Finally, we implemented real-time crosslinking reaction monitoring strategies to understand reaction kinetics and support the optimization

of reaction conditions for future large-scale manufacturing. The TNBSA assay revealed a gradual decline in free amines, reaching near-complete consumption at ~ 400 min (Fig. 2G). Simultaneously, pH steadily dropped, plateauing at 6.4 after the same time point, consistent with the progression of amidation. Viscosity measurements revealed a moderate rise during initial crosslinking, followed by a sharp increase after 500 min (Fig. 2H). This late-stage viscosity surge, despite stable amine levels, indicated ongoing esterification reactions between the hydroxyls of HA and the carboxylates of the polymer (a known side reaction [72,75]). To prevent undesired network overgrowth and ensure reproducibility, we defined 400 min as the optimal endpoint for synthesis.

While we monitored HA-CP crosslinking via pH and viscosity and controlled for impurities and yields across batches (Fig. S22), we acknowledge that ester bond formation between HA carboxyl groups and polyol hydroxyl groups may occur as a side reaction under mildly acidic conditions. Although prior reports suggested a minimal and reversible impact [29], the potential impact of esterification on mucoadhesion and degradation kinetics warrants further study. Future work will include structural characterization to quantify ester content and assess functional consequences.

With a view toward industrial-scale production of HA-CP, we carried out scaled-up experiments for the DMTMM reagent and the final HA-CP product, generating multiple batches and recording the starting amount (in grams) of the primary reagent and the final product (Fig. S22A–C). We achieved manufacturing yields of 95 % for DMTMM, resulting in an average of 700 g of product per batch, and yields of approximately 95 % for HA-CP, producing around 1300 g per batch. We evaluated critical characterization parameters to ensure product conformity, including viscosity and the percentage of residual synthesis reagents (N-methylmorpholine [NMM], acetone, and methanol). The results demonstrated robust batch-to-batch consistency within the studied variability range for viscosity (Fig. S22A–C), with residual by-product levels below 0.06 % w/w, well under the total impurity limits specified by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [76].

HA-CP also demonstrated greater enzymatic stability compared to unmodified HA; in degradation assays using hyaluronidase treatment and SEC-RI-MALS analysis, HA degraded more rapidly than HA-CP, which retained its Mw profile longer (Fig. S23). Bimodal Mw distributions indicated a protective effect of the crosslinked matrix, due to steric hindrance limiting enzyme access. This property enhances HA-CP's suitability for mucosal delivery, where enzymatic degradation can compromise native HA performance. The optimization of both individual components facilitated the straightforward preparation of the hybrid biomaterial via physical powder mixture and aqueous reconstitution. Fig. 1G represents an example, StPGA-SS-CL embedded in HA-CP, suitable for intranasal administration. TEM images (Fig. 1H) clearly depict the presence of both components with a clear interaction between the HA-CP hydrogel fibers and the StPGA-SS-CL nanocarrier. Further TEM and Cryo-TEM images (Fig. S24) reveal apparent differences for

Table 2
Physicochemical characterization of HA-CP.

Comp.	Diffusion Coefficient ($\text{m}^2 \text{s}^{-1}$)			Hydrodynamic Radius (nm)			Mw (kDa) ^a	PDI ^a	Mw kDa ^b
	HA	PGA	Lys	HA	PGA	Lys			
HA	$1.8 \cdot 10^{-11c}$	–	–	11.1	–	–	49.1	1.12	67
LinPGA	–	$7.4 \cdot 10^{-11c}$	–	–	2.7	–	18.9	1.37	–
Lys	–	–	$5.1 \cdot 10^{-10c}$	–	–	0.4	–	–	–
Physical Mixture	$1.9 \cdot 10^{-11}$	–	$1.6 \cdot 10^{-9}$	10.4	–	0.1	–	–	–
HA-CP	$1.1 \cdot 10^{-11}$	–	^d	17.9	–	^d	145.2	1.86	114

^a Obtained by SEC.

^b Obtained by intrinsic viscosity.

^c Values determined at 15 mg/mL.

^d Lys Signals in HA-CP not used for DOSY NMR calculations due to very low intensity. Abbreviations: HA = hyaluronic acid; HA-CP = hyaluronic acid-poly-L-glutamate cross-polymer; LinPGA = linear poly-L-glutamate; Lys = lysine; Mw = molecular weight.

nanocarriers in the presence and absence of HA-CP, demonstrating the robustness of our platform among the different nanocarriers used.

In summary, we developed and optimized a scalable synthetic procedure for HA-CP production, yielding a hydrogel with robust physicochemical properties and reproducible batch-to-batch characteristics suitable for large-scale manufacturing. Importantly, monitoring the crosslinking reaction closely enabled precise control and process optimization; furthermore, HA-CP demonstrated an increased resistance to enzymatic degradation compared to native HA, underscoring its potential for sustained intranasal delivery applications.

2.2. Mucosal penetration studies of platform components in ex vivo and in vitro models

We employed a vertical Franz diffusion cell (Fig. S25) to simulate the air-exposed environment of the nasal mucosa; this apparatus, the only standardized method cited in pharmacopeias, offers advantages such as small sample volume requirements (300 μ L) over horizontal systems. We selected excised ovine nasal mucosa for its close resemblance to human tissue in terms of Mw transport and metabolic/transporter profiles [77]. We applied OG-labeled PGA derivatives (developed previously) to the donor chamber, with isopropyl alcohol (iPrOH) employed as a negative control, and collected samples from the receptor chamber over 24 h to quantify OG diffusion. Fig. 3A1 depicts the cumulative permeation profiles; although cumulative delivery remains informative, we emphasize permeation rate in this ex vivo model, as the Franz cell lacks mucociliary clearance and dynamic fluid flow (critical features in vivo). Table S3 lists all formulations evaluated.

In a physiological context, faster permeation enhances drug delivery efficiency by minimizing clearance and improving access to the olfactory region. Accordingly, we used the permeation rate as a proxy for therapeutic performance in nasal delivery. Initial comparisons between LinPGA and StPGA (both with similar Rh values of 10–13 nm, no aggregation, and random coil conformation; Table 1, Fig. S14) revealed that StPGA achieved a more rapid permeation rate, despite similar 24-h cumulative permeation (Fig. 3A2). NMR confirmed the absence of OG cleavage by mucosal enzymes within 6 h (Fig. S26). Based on this performance, we selected StPGA as the scaffold for further functionalization.

We next compared crosslinking strategies. StPGA-CL, with increased Rh (90 nm), displayed slower and reduced permeation relative to unmodified StPGA (10 nm) (Fig. 3A3). In contrast, StPGA-SS-CL (Rh 200 nm) achieved both higher permeation rate and cumulative delivery than StPGA and StPGA-CL. These results indicate that factors beyond size (i.e., surface chemistry and mucoadhesiveness) critically influence transport. The thiol-rich disulfide bonds in StPGA-SS-CL likely enhance permeation through thiol/disulfide exchange with mucin cysteine residues [78]; this mechanism, validated in thiolated polymers such as chitosan, poly(acrylic acid), carboxymethylcellulose, and alginates, has supported up to 140-fold increases in mucoadhesion [37]. Thus, disulfide crosslinking enhances both mucosal interaction and permeability, unlike conventional click-crosslinked StPGA-CL, where increased size hinders transport. Functionalization with OL emphasized the importance of ligand orientation. Although StPGA-OL(C-term) and StPGA-OL(N-term) share similar structures, only the C-terminal variant significantly improved nasal permeation at 24 h (Fig. 3A4). This finding suggests that adequate spatial presentation, rather than ligand presence alone, dictates receptor-mediated uptake. C-terminal conjugation likely preserves OL's native conformation, facilitating effective binding to fucosylated receptors in the nasal epithelium. In contrast, N-terminal attachment may obscure key residues or alter structure, limiting receptor interaction [40]. These findings are consistent with prior studies, which have demonstrated that anchoring position affects ligand binding, kinetics, and downstream signaling [79,80].

StPGA-HA80kDa exhibited enhanced permeation kinetics and cumulative delivery compared to unmodified StPGA; in contrast, the

physical mixture of StPGA and HA (StPGA/HA80kDa) demonstrated reduced diffusion (Fig. 3A5), likely due to increased viscosity or transient intermolecular entanglements that hinder mucosal barrier passage. Interestingly, the hybrid formulation StPGA/HA-CP initially displayed slower permeation, suggesting transient mucosal retention, but achieved cumulative delivery comparable to StPGA-HA80kDa by 24 h (Fig. 3A5). These findings support the function of HA-CP as a “depot-like enhancer,” forming a viscoelastic matrix at the administration site that facilitates controlled, sustained release rather than promoting rapid diffusion [81,82]. This effect is consistent with our previous findings [71], where HA-CP enhanced tissue penetration and sustained release of PGA-drug conjugates in skin models. Notably, despite its delayed onset, StPGA/HA-CP achieved 100 % cumulative permeation at 24 h, outperforming unmodified StPGA (79 %), and thereby underscoring its potential for extended intranasal delivery.

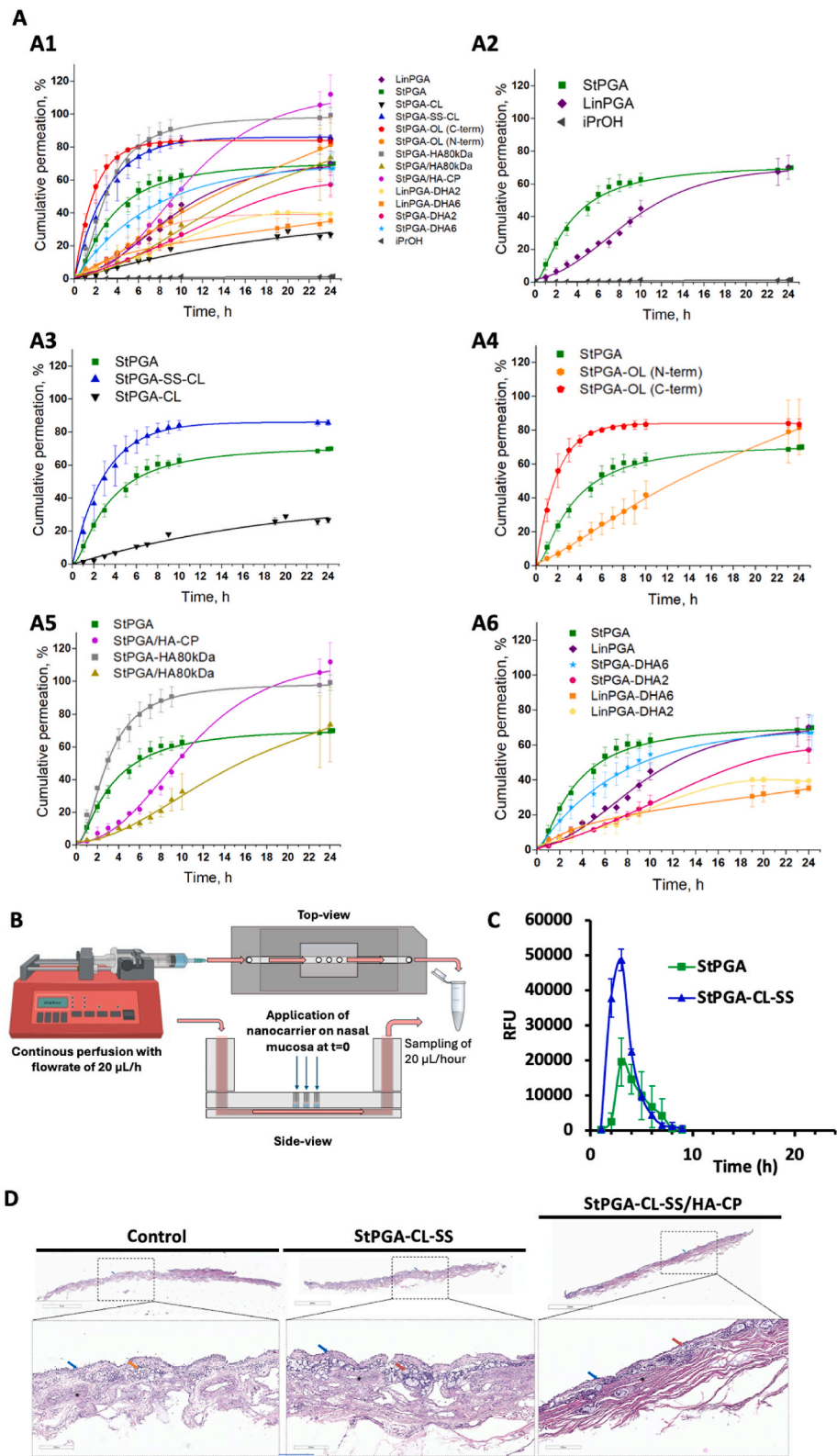
Further investigation into DHA conjugation revealed that StPGA-DHA6 and StPGA-DHA2 outperformed their linear analogs (LinPGA-DHA6 and LinPGA-DHA2) in cumulative permeation (Fig. 3A6); however, none exceeded the performance of unmodified or HA-modified StPGA, likely due to aggregate formation (Table 1, Fig. S18). Aggregation, driven by DHA's lipophilicity, may confine the conjugated moieties within the particle core, limiting mucosal interaction and reducing mucodiffusivity [70]. Additionally, low degrees of DHA modification promoted point-to-point aggregation and increased particle size, further impairing permeation. This size increase is characteristic of polyelectrolytes conjugated to hydrophobic drugs at low substitution levels [83]. Based on these limitations, we excluded DHA-conjugated formulations from further development.

Ex vivo Franz diffusion cell studies identified three primary factors governing nasal mucosal transport: (i) polymer architecture, where StPGA enabled faster permeation than its linear counterpart (LinPGA); (ii) particle size, with larger carriers exhibiting reduced permeation unless compensated by mucodiffusive design; and (iii) surface chemistry, particularly the role of ligand exposure and mucoadhesive interactions in determining transport dynamics.

Based on these insights, we selected three lead formulations for in vivo testing in murine models: StPGA-OL(C-term), offering the fastest permeation and receptor-targeting potential; StPGA-CL-SS, employing thiol-mediated mucosal adhesion to enhance permeation despite increased size and unmodified StPGA, serving as a reference to benchmark the influence of branched architecture.

While StPGA-HA80kDa exhibited favorable behavior, we prioritized StPGA/HA-CP for further evaluation due to its formulation simplicity, scalability, and depot-like enhancement, which resulted in the highest 24 h accumulation. These sustained release properties are particularly advantageous for nose-to-brain drug delivery. The integrated platform, combining PGA-based nanocarriers with HA-CP, thus offers a scalable and modular system for intranasal transport of diverse therapeutic payloads.

Despite their widespread use, Franz diffusion cells remain limited by their static nature, lacking features of the nasal environment such as mucociliary clearance, airflow, and enzymatic activity; these omissions may lead to overestimated permeation and stability, limiting predictive power. To address this problem, we developed a nasal-mucosa-on-a-chip (NMoC) platform incorporating primary human nasal epithelial cells (HNEPCs) cultured at the air-liquid interface (ALI) and continuously perfused at 20 μ L/h (Fig. 3B). This microfluidic system mimics mucosal clearance, enables real-time apical-to-basal sampling, and supports dynamic kinetic profiling under physiologically relevant conditions. At $T = 0$, we applied a 2 μ L bolus of Cy5.5-labeled nanocarriers (10 mg/mL) to the apical compartment and collected effluent samples (20 μ L) hourly. The NMoC architecture comprises defined flow paths, culture wells, and sampling reservoirs. Fluorescence quantification revealed distinct kinetic profiles: StPGA displayed a delayed T_{max} (~3 h) followed by gradual decline, while StPGA-SS-CL, despite a larger Rh (200 vs. 10 nm), displayed an earlier T_{max} (~2 h) and higher cumulative permeation



(caption on next page)

Fig. 3. Ex vivo and in vitro permeation studies of PGA-based formulations. **(A)** Panel A1 depicts the comparative ex vivo permeation profiles of OG-labeled PGA-based formulations employing the Franz diffusion cell with bovine nasal mucosa. This figure is further divided into experimental groups as follows: A2 - LinPGA and StPGA and isopropanol (iPrOH) as negative control; A3 - StPGA and crosslinked systems prepared via azide-alkyne cycloaddition (StPGA-CL) or disulfide crosslinkers (StPGA-CL-SS); A4 - StPGA modified with OL via the C-terminus (StPGA-OL(C-term)) and N-terminus (StPGA-OL(N-term)); A5 - StPGA-HA80kDa, the physical mixture of StPGA and HA80kDa (StPGA/HA80kDa), and StPGA embedded in HA-CP (StPGA/HA-CP); and A6 - LinPGA and StPGA DHA conjugates with low (LinPGA-DHA2/StPGA-DHA2) and high (LinPGADHA6/StPGA-DHA6) degrees of modification. Data presented as mean $\pm$ s.d., $n = 3$. **(B)** Nasal-Mucosa-on-Chip (NMoC) scheme depicting the microfluidic platform in continuous perfusion and the top and side views of the chip design. **(C)** StPGA and StPGA-CL-SS were applied to each microtissue well containing the air-liquid interface (ALI)-cultured nasal epithelium, and fluorescence was measured over time up to 24 h. **(D)** H&E staining of the nasal mucosa tissue after 4 h permeation studies in the Franz diffusion cells treated with control (PBS), StPGA-CL-SS, and StPGA-CL-SS/HA-CP. As indicated in the figure, the original magnification displayed in the upper images was 4 mm, while the magnified images below used 500 nm. One representative image shown for each treatment.

(Fig. 3C). These results closely mirrored those observed in Franz diffusion cells (Fig. 3A3), validating the NMoC's physiological relevance. Notably, the two systems revealed divergent delivery kinetics: the Franz cell, devoid of clearance mechanisms, revealed monotonic cumulative permeation over 24 h, favoring formulations like StPGA-SS-CL, while the NMoC revealed biphasic, non-linear kinetics, with rapid initial uptake followed by decay, more representative of in vivo dynamics. In the NMoC, perfusion at 20 μ L/h simulated mucociliary clearance, generating a transient exposure window. Moreover, the ALI-cultured epithelial barrier further regulated transport, which is absent in static Franz cells. These observations underscore a conceptual distinction: Franz cells estimate theoretical permeation under static conditions, whereas the NMoC captures dynamic transport constrained by biological factors. While both platforms provide valuable insights into permeability and formulation behavior, neither fully replicates nasal physiology, including regional deposition, immune responses, and complex airflow; as a consequence, in vivo validation remains essential.

Both models consistently ranked StPGA-SS-CL above StPGA, confirming the robustness of the observed trend; however, only the NMoC revealed transient kinetics critical to nose-to-brain delivery, where formulations must rapidly penetrate before clearance. The NMoC's ability to replicate dynamic clearance provided an enhanced IVIVC, as supported by in vivo data (Fig. 4). While useful for early screening, the limited temporal resolution of the Franz cell precludes the assessment of rapid pharmacokinetics; the NMoC, by contrast, enables refined, physiologically informed nanocarrier evaluation. By integrating ALI exposure, perfusion, and epithelial barriers, the NMoC supports assessment of both passive diffusion and active mucosa–nanocarrier interactions. The miniaturized design and high sensitivity also make the NMoC well-suited for early-stage formulation development with limited material. Overall, real-time, pharmacokinetic-like data can enhance the mechanistic understanding of mucosal transport.

The NMoC represents a scalable, predictive tool for intranasal drug delivery, providing both physiological relevance and high-resolution kinetic profiling. The concordance with Franz cell-based formulation rankings, coupled with an ability to detect dynamic permeation kinetics, underscores the utility of the NMoC platform in the preclinical development of CNS-targeted therapeutics, biologics, and mucosal vaccines. Future enhancements, such as mucus-mimetic coatings, immune cell integration, and computational fluid dynamics, will further increase model fidelity. Finally, the enhanced performance of StPGA-SS-CL, attributed to disulfide-mediated mucoadhesion, highlights the pivotal role of surface-engineered functionality in the design of effective intranasal nanocarrier systems.

2.3. Primary culture cell viability studies and histological evaluation confirm the safety of nanocarrier and hybrid biomaterials

To comprehensively assess the safety profile of the developed nanocarriers and HA-CP formulation, we performed a multi-tiered evaluation using in vitro, ex vivo, and in vivo models.

To assess mucosal integrity following nanocarrier exposure, we performed histological analysis of nasal mucosal tissue harvested from Franz diffusion cell experiments. We stained tissues with haematoxylin

and eosin (H&E) to evaluate epithelial preservation, glandular morphology, and vascular architecture. In control conditions (Fig. 3D, left panel), the epithelial lining (blue arrow) remained intact along the full length of the membrane, appearing as a pseudostratified layer. The underlying lamina propria exhibited numerous mucosal glands (red arrow) with clear nuclear architecture, embedded in a well-vascularized stroma (asterisk). These features represent the normal histological appearance of ex vivo nasal mucosa. In tissue exposed to StPGA-SS-CL-Cy5.5 for 4 h (Fig. 3D, middle panel), histological preservation remained comparable to controls. We observed a maintained epithelial barrier without disruption or thinning, while the mucosal glands displayed viable nuclei and an intact structure. We observed no signs of inflammatory infiltration, epithelial detachment, or stromal damage. Similarly, exposure to StPGA-SS-CL-Cy5.5 in combination with HA-CP (4 h) (Fig. 3D, right panel) did not induce any detectable tissue damage. The epithelium remained continuous, and glandular architecture and vascular elements appeared unaltered. These findings confirm that the combination formulation maintains biocompatibility under experimental conditions relevant to permeation studies. Overall, the absence of morphological alterations across all conditions indicates that StPGA-SS-CL with and without formulation with HA-CP remains non-toxic and well-tolerated by the nasal mucosa following short-term exposure. This finding agrees with the absence of cell toxicity observed when we evaluated nanocarriers in the primary microglia cells (S1.2 and Fig. S27A) and the low lactate dehydrogenase (LDH) release observed in the NMoC model from HNEPCs (Fig. S27B), further supporting the safety profile of these nanoformulations for intranasal administration.

To expand on mucosal tolerability, we conducted a vaginal irritation assay in rabbits using HA-CP 1 % w/v (Fig. S28 and Table S2). Daily administration over 5 days produced no signs of erythema, edema, or tissue damage. Histological evaluation revealed intact epithelial barriers and normal stromal morphology, and a vaginal irritation index of 0 confirmed excellent mucosal compatibility per ISO guidelines (S1.2).

To ratify our ex vivo findings, we next employed BALB/c mice as an in vivo model (Fig. 4), given their extensive use in intranasal drug delivery and pharmacokinetic studies, thanks to a well-characterized immune and olfactory systems, availability, and reproducibility. While we acknowledge interspecies anatomical differences (particularly the proportionally larger olfactory region in rodents compared to humans), BALB/c mice provide a consistent and ethically feasible model for evaluating nasal absorption and potential nose-to-brain transport. We performed immunohistochemical analysis of CNS tissue to evaluate potential neuroinflammation, examining the olfactory bulb and cortex for Iba1, a marker of microglial activation, following intranasal administration (Fig. S29). High-resolution confocal tile-scan images and quantification revealed no increase in Iba1 signal intensity in animals treated with StPGA-CL-SS or StPGA-OL(C-term), with or without HA-CP, compared to PBS controls. High-magnification images ($63\times$) revealed that microglia retained their ramified, resting morphologies, without evidence of amoeboid transformation, indicating a lack of activation of neuroimmune responses under the tested conditions. Complementary confocal immunofluorescence imaging (Figs. S30–S31) failed to reveal glial fibrillary acidic protein (GFAP) upregulation in astrocytes and/or localized aggregation or inflammatory responses in Iba1-positive

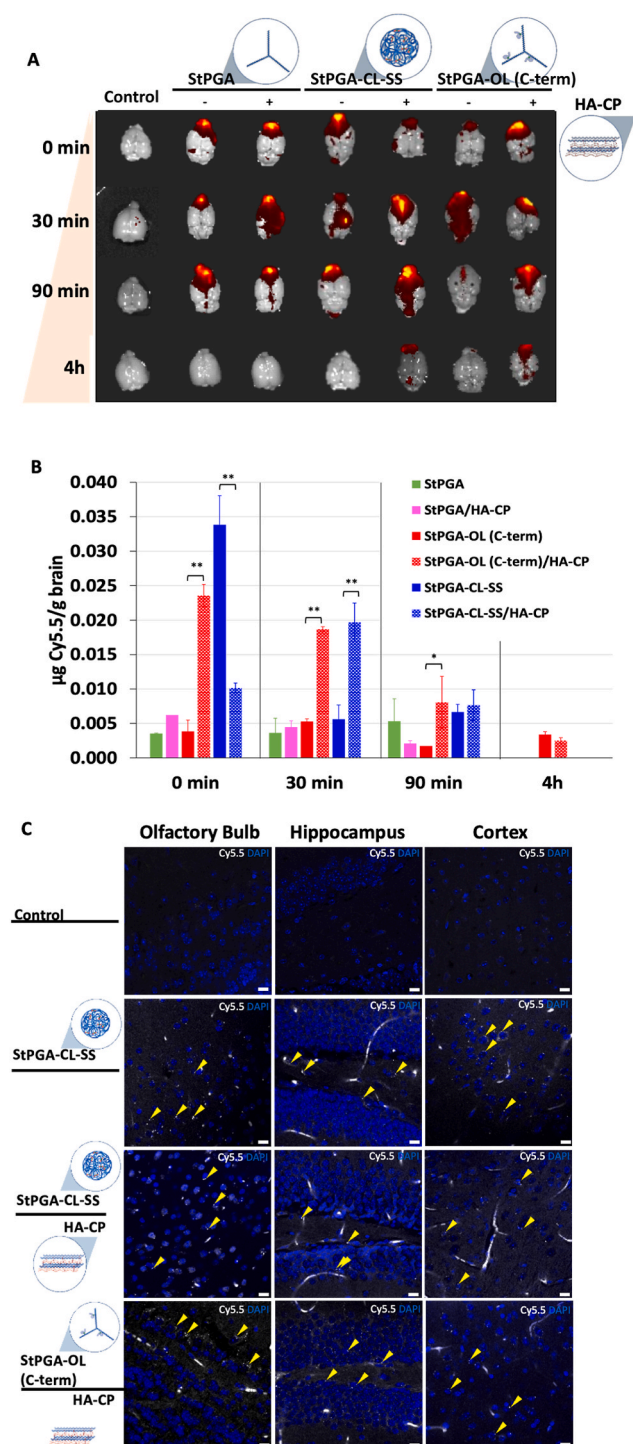


Fig. 4. Biodistribution studies of Cy5.5-labeled PGA-based nanocarriers with/without HA-CP after intranasal administration in mice. **(A)** Representative ex vivo fluorescence images of the dissected whole brain after intranasal administration of Cy5.5-labeled PGA-based formulations to healthy mice: StPGA and StPGA/HA-CP; StPGA-SS-CL and StPGA-CL-SS/HA-CP; and StPGA-OL(C-term) and StPGA-OL (C-term)/HA-CP. **(B)** Quantification of Cy5.5 fluorescence after brain homogenization. Data expressed as µg of dye per g of tissue ($n > 4$, mean $\pm$ SEM). **(C)** Confocal microscopic images of brain tissue sections 30 min after intranasal administration of PBS (control), Cy5.5-labeled StPGA-CL-SS with and without HA-CP, and StPGA-OL(C-term) with HA-CP. Cy5.5 signal labeled in white, and cell nuclei stained with DAPI shown in blue. Arrows indicate representative Cy5.5-labeling. Scale bar = 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

microglia. Furthermore, we detected Cy5.5-labeled nanocarriers within the olfactory mucosa and parenchyma with expected distribution and no signs of tissue reactivity, suggesting both permeability and safety of the formulations.

Taken together, these complementary datasets demonstrate the tolerability of StPGA-based nanoconjugates and the HA-CP hydrogel in relevant CNS and mucosal environments. While additional toxicological assessments (e.g., cytokine release and behavioral endpoints) may be warranted for future clinical translation, the absence of cellular toxicity, structural disruption, or neuroinflammation in these studies provides a strong foundation for ongoing preclinical development of these formulations for nose-to-brain delivery.

2.4. In vivo biodistribution of selected intranasal nanocarrier systems

To enable precise monitoring and quantification of nasal barrier crossing by the nanocarriers in vivo, we covalently labeled selected formulations with Cy5.5. We intranasally administered Cy5.5-labeled StPGA, StPGA-OL(C-term), StPGA-CL-SS, and their HA-CP formulations to healthy BALB/c mice at eq. Cy5.5 doses (30 mg/kg) to evaluate biodistribution (intranasally administering PBS to the control group). We then euthanized animals at designated time points post-administration (from 0 min to 4 h), collected blood, and then harvested major organs (brain, lungs, liver, spleen, kidneys, heart) for ex vivo analysis via IVIS® ex vivo fluorescence imaging to determine biodistribution and percentage of the administered dose (% AD). In addition, we histologically examined brain sections to assess regional localization and cellular internalization of the nanocarriers.

Ex vivo imaging data confirmed successful nose-to-brain transport for all candidates within 90 min (Fig. 4A), with initial localization to the olfactory bulbs (primary entry point for intranasally delivered agents to the CNS) followed by gradual migration toward posterior brain regions (Fig. 4A). This spatiotemporal pattern reflects a typical distribution pathway for nanoconstructs that utilize the olfactory and trigeminal neural routes. Among the tested candidates, StPGA-CL-SS/HA-CP demonstrated the most extensive brain-wide diffusion, likely due to synergistic effects of thiol-mediated mucoadhesion (via disulfide-thiol exchange with mucin cysteines) and sustained release from the HA-CP matrix. The disulfide-functionalized carrier may transiently adhere to mucus, increasing residence time, while the HA-CP component slows the release and prolongs the presence of the nanocarrier at the administration site, enabling more complete uptake. In contrast, carriers administered without HA-CP displayed faster initial penetration but more restricted localization, predominantly within the olfactory bulb region.

The co-delivery of StPGA-OL(C-term) with HA-CP prompted delayed but sustained brain accumulation, consistent with a depot-like release profile (Fig. 4A and B); this mixture displayed detectable fluorescence signal retention in brain tissue up to 4 h after treatment, exceeding StPGA to a greater extent than StPGA/HA-CP. The prolonged signal retention likely reflects a major mucoadhesive property due to ligand-mediated receptor targeting (via L-fucose binding by OL) compared to the more mucodiffusive character observed for StPGA-SS-CL. This mechanism could enhance targeting precision and exposure duration if required. Cy5.5 quantification in brain homogenates (Fig. 4B) demonstrated a higher amount of StPGA-CL-SS in the brain immediately after administration (0 min) compared to other formulations; however, StPGA-CL-SS/HA-CP and StPGA-OL(C-term)/HA-CP demonstrated the highest accumulation at 30 min, displaying significant differences compared to StPGA-CL-SS and StPGA-OL(C-term) without HA-CP. We still observed the depot effect generated by HA-CP after 90 min, with significant differences between StPGA-OL(C-term) with and without HA-CP. Together, these findings reinforce the importance of formulation strategy in modulating both the kinetics and extent of nose-to-brain transport. We quantified Cy5.5 signal via fluorescence spectroscopy following organic solvent extraction from tissue homogenates. Calibration curves generated from spiked tissue samples demonstrated high

linearity ($R^2 > 0.998$), and we employed non-treated organs as negative controls to correct for autofluorescence. The method yielded a limit of detection (LoD) of 0.000164 $\mu\text{g/mL}$ and a limit of quantification (LoQ) of 0.000496 $\mu\text{g/mL}$. While fluorescence spectroscopy provided sufficient sensitivity and specificity for this study, future work will incorporate HPLC-based methods to further validate quantification accuracy in complex biological matrices.

We measured a nasal residence time of our platform of approximately 4 h; this duration lies within the range reported for effective intranasal delivery systems that support therapeutic outcomes, particularly when combined with enhanced mucosal permeation and brain targeting, as well as from the data obtained with the NMoC model (Fig. 3C). Moreover, HA-CP provides a depot-like environment that sustains local concentration and modulates release kinetics, potentially extending the effective exposure window beyond the physical residence time. While longer retention may be beneficial for chronic dosing, our results suggest that even short-term residence enables efficient delivery to the brain. Future studies will explore formulation strategies to further prolong nasal retention and evaluate therapeutic efficacy in chronic administration models.

We also assessed total organ biodistribution by ex vivo fluorescence imaging (Fig. S32) and quantitative fluorescence analysis following tissue homogenization (Table S4). Quantitative analysis of all homogenized organs revealed brain accumulation levels of 2.4 % of AD/g tissue for StPGA-OL/HA-CP and 2.2 % AD/g tissue for StPGA-CL-SS/HA-CP at 30-min post-administration (Fig. 4B and Table S3). While lower than anticipated, these values reflect practical limitations such as oral capillary absorption during intranasal administration, as evidenced by concurrent intestinal fluorescence (Fig. S32 and Table S4). While this study demonstrated nose-to-brain delivery and regional brain distribution of the nanocarriers, the fate of transported elements within brain tissue remains incompletely elucidated. Given the biodegradable nature of the platform components (e.g., PGA, disulfide linkers, and natural ligands), we anticipate that nanocarriers undergo enzymatic and redox-mediated degradation; however, future studies will be necessary to directly assess the clearance, metabolic breakdown, and potential accumulation of nanocarriers in the brain, particularly when under chronic dosing conditions.

Histological analysis of brain tissue from the best-performing experimental conditions (as previously described) revealed intracellular localization of StPGA-CL-SS (both alone and in combination with HA-CP) and StPGA-OL(C-term) delivered with HA-CP (Fig. 4C). We detected fluorescent signals in all three brain regions studied: the olfactory bulb, the dentate gyrus of the hippocampus, and the somatomotor cortex (Fig. 4C). Although the observations remain qualitative, they suggest that the delivery systems enable efficient penetration into the brain and widespread diffusion across distinct anatomical areas. Fluorescence-based biodistribution analysis confirmed successful delivery to brain tissue, with preferential accumulation observed in the olfactory bulbs and frontal cortex (regions anatomically linked to direct nose-to-brain transport). These findings agree with known pathways of intranasal delivery via the olfactory pathway; however, further studies using high-resolution imaging techniques (e.g., fluorescence microscopy of brain sections or mass spectrometry imaging) will be necessary to fully characterize the spatial distribution and cellular localization of the nanocarriers within the CNS. The fluorescent signal revealed a predominantly intracellular distribution, characterized by a punctate pattern concentrated around the nucleus, with no signal detected within nuclear compartments. We most frequently observed signals in the cytoplasm of microglial cells and neurons (Fig. S30) and, to a lesser extent, in astrocytes (Fig. S31). Additionally, we noted Cy5.5 fluorescence along the walls of blood vessels, suggesting uptake by endothelial cells and associated astrocytic processes. This perivascular pattern may indicate early stages of parenchymal entry or specific interactions with the neurovascular unit. Importantly, we failed to observe this fluorescence pattern in brain sections from vehicle-treated control animals,

confirming the specificity of the observed labeling (Figs. S30–31).

In conclusion, our in vivo biodistribution studies provided evidence that rationally engineered PGA-based nanocarriers, particularly when combined with the depot-forming HA-CP matrix, can traverse the nasal mucosa and achieve regionally distributed brain delivery. The brain accumulation observed for StPGA-CL-SS/HA-CP and StPGA-OL(C-term)/HA-CP formulations demonstrates how structural features (e.g., thiol-mediated mucoadhesion and ligand exposure) can synergize with sustained-release strategies to improve CNS targeting. The observed intracellular localization in microglia, neurons, and endothelial cells across brain regions further reveals the ability of these formulations to reach both parenchymal and neurovascular compartments. Despite systemic distribution and off-target intestinal uptake, likely due to unavoidable swallowing during intranasal dosing, the overall signal specificity and retention in brain tissue highlight translational potential.

These findings gain further significance when contrasted with previously reported lipidic or polymeric systems, which often exhibit poor retention, limited access to deep brain regions, or inconsistent targeting due to passive diffusion and rapid mucociliary clearance [7,9,10,33,84]. In contrast, our system achieves sustained brain presence at both 30 min and 4 h post-administration without relying on PEGylation, absorption enhancers, or cationic modifications, which constitute commonly employed factors in earlier platforms often associated with scalability and tolerability concerns [17,46,58]. The combined architecture of StPGA-based nanoconjugates embedded in the HA-CP matrix constitutes a modular, biocompatible, and reproducible platform. Our design integrates three complementary strategies: (i) biodegradable disulfide crosslinking for redox-sensitive release and thiol-mediated mucoadhesion; (ii) site-specific ligand conjugation (e.g., OL) for targeted uptake; and (iii) a viscoelastic HA-CP matrix to prolong nasal residence and enhance upper-cavity deposition.

This design contrasts with most reported intranasal systems, which tend to focus either on mucoadhesion or targeting, but rarely both. For example, thiolated chitosan and polyacrylic acid derivatives improve mucoadhesion by up to 140-fold [35], supporting our rationale for covalent disulfide crosslinking. Likewise, lipid or polymeric nanoparticles with lectin-like ligands can enhance receptor-mediated uptake [38–40] but often rely on post-synthetic surface modifications or non-covalent adsorption, which compromises stability and reproducibility. Recent reviews [11,85–87] typically classify intranasal systems by material type, lipids, nanostructured carriers, or mucoadhesive hydrogels, without emphasizing hybrid systems that integrate multiple delivery mechanisms; for instance, Chen et al. highlighted liquid sprays such as NEO100 [11]; Wang et al. reviewed polysaccharide nanogels lacking depot functionality [86]; and Wu et al. did not address multifunctional, matrix-embedded nanocarriers [87]. To our knowledge, no prior study has reported the combined implementation of disulfide-crosslinked polypeptide nanocarriers embedded within a viscoelastic HA-CP mucosal depot for CNS delivery.

Compared to earlier polypeptide- or HA-based systems, our formulation offers several distinct advantages; i) the avoidance of PEGylation and cationic surfactants, thereby improving safety and regulatory compatibility; ii) the inclusion of the disulfide-crosslinked StPGA backbone enables redox-triggered release and durable covalent mucoadhesion; iii) site-specific ligand conjugation allows tunable targeting without the need for post-synthetic modification; and iv) the HA-CP matrix (already used in commercial dermal formulations - INCI: Hyaluronic Acid/Polyglutamic Acid Crosspolymer; EU CosIng ID 93223 [<https://ec.europa.eu/growth/tools-databases/cosing/details/93223>]) further improves nasal residence time and delivery efficiency.

Although lipid-based systems remain the benchmark, our polypeptide platform provides greater modularity, chemical precision, and functional stability. This composite system, merging nanocarrier design, covalent mucoadhesion, ligand-mediated targeting, and depot-enhanced delivery, represents a scalable, biocompatible, and translationally relevant solution for intranasal CNS therapy. Nevertheless,

challenges remain. While biodistribution confirms regional brain delivery, the efficiency is consistent with typical intranasal bioavailability levels (<1–5%) [11]. Additional studies will address biological variability, chronic safety of disulfide linkages and ligands, and long-term mucosal interactions. Although Franz diffusion and NMoC assays support our proposed mechanisms, direct quantification of mucoadhesion (e.g., via rheology, tensile testing, or quartz crystal microbalance) will further validate performance.

From a regulatory perspective, PGA and HA have established safety profiles and precedents in clinical and cosmetic applications, which may facilitate regulatory progression. PGA is a biodegradable and non-immunogenic polypeptide, while HA is extensively used in injectable and topical products. The aqueous-based, solvent-free synthesis displays compatibility with good manufacturing practices standards and enables reproducible, scalable production. Intranasal delivery also offers a non-invasive route suitable for chronic use, and the HA-CP matrix's viscoelasticity supports formulation into patient-friendly formats such as nasal gels or sprays.

Collectively, these attributes support the translational potential of this platform (NanoInBrain) by integrating regulatory familiarity, manufacturing scalability, and patient usability. Future efforts will focus on optimizing ligand density, evaluating chronic safety, and developing adaptable formats, including responsive hydrogels and artificial intelligence-guided tuning of delivery.

3. Conclusion

Our rationally designed, modular platform for non-invasive intranasal delivery to the brain integrates tunable PGA-based nanocarriers with a viscoelastic HA-CP matrix to overcome mucosal barriers and sustain CNS exposure. Through the strategic control of nanocarrier architecture, disulfide-based crosslinking, and covalent ligand conjugation, we have developed a scalable and biocompatible system that addresses the major bottlenecks of intranasal delivery, including permeation, retention, and targeting.

Specific design features represented critical aspects linked to performance. Branched PGA structures enhanced mucosal transport compared to linear analogs, while disulfide crosslinking (StPGA-CL-SS) conferred redox-responsive release and thiol-mediated mucoadhesion, thereby increasing residence time and diffusion. Site-specific C-terminal conjugation of targeting peptides preserved receptor affinity, enabling regional delivery to brain targets. Embedding the nanocarriers into an HA-CP matrix prolonged nasal residence, promoted upper-cavity deposition, and supported formulation reproducibility. This unique combination of matrix embedding and multifunctional nanocarrier design had remained unreported in recent CNS-focused delivery systems.

The NMoC model enabled high-resolution kinetic profiling under physiologically relevant clearance conditions, revealing a strong correlation with ex vivo and in vivo findings. In vivo biodistribution studies demonstrated sustained signal across the olfactory bulb, cortex, and hippocampus, with intracellular localization in neurons, microglia, and endothelial cells. Complementary histology and Iba1-based immunostaining confirmed local safety at the tested dose and time point, supporting the tolerability of acute administration. Together, these data provide multiscale validation of the platform's targeting and safety profile.

Positioned within the evolving landscape of CNS drug delivery, this system unites structural modularity, ligand-level targeting, and depot-mediated mucosal retention into a single biodegradable and adaptable architecture. The platform holds promise for treating neurodegenerative disorders, neuroinflammation, and CNS malignancies via a patient-friendly administration route. Future directions will focus on integrating therapeutic payloads, ensuring the safety of chronic dosing, and developing sprayable or responsive hydrogel formats, thereby paving the way for precision neurotherapeutics through intranasal delivery.

4. Materials and methods

4.1. Materials

Supplementary Information (SI) includes a comprehensive list of materials and ethical approvals (Section S1.1). Section S1.2 provides detailed experimental protocols for the synthesis, labeling, and physicochemical characterization of all newly developed PGA-based nanocarriers (StPGA-CL-SS, StPGA-OL, StPGA-HA, and DHA-PGA), as well as the HA-CP hydrogel. This includes reagent specifications, coupling strategies, purification methods, and fluorescence quantification techniques. Section S1.3 contains all Supplementary Figures and Tables (Tables S1–S4), ranging from structural and analytical data (Figs. S1–S24) to additional biodistribution and immunohistochemistry results (Figs. S25–S32).

4.2. Methods

4.2.1. Synthesis and characterization of PGA-based nanocarriers

A series of novel polypeptide-based nanocarriers was synthesized, including StPGA-CL-SS, StPGA-OL, StPGA-HA, and DHA-modified PGA constructs. Except for PGA-DHA conjugates [70], these architectures were newly designed for this study to provide structural tunability, redox-responsiveness, ligand targeting, and mucoadhesive functionality. Synthetic routes included copper- and disulfide-mediated crosslinking, peptide (odorranalectin) and lipid (DHA) conjugation, and covalent coupling to hyaluronic acid (HA) via carbodiimide chemistry. All synthetic protocols and characterization data are provided in the Supplementary Information (Sections S1.2–S1.3; Figs. S1–S24), including ¹H-NMR, SEC, DLS, CD, Cryo-TEM, and fluorescence measurements.

4.2.1.1. Fluorophore labeling. Oregon Green (OG): PGA-acid derivatives were labeled in DMF using OG-cadaverine (0.009 eq. GAUs), DIC (0.03 eq.), and HOBt (0.03 eq.) for 48 h. After precipitation and dialysis (MWCO 0.5–1 kDa), the product was purified by Sephadex® G25. OG loading: 1.5–2.4 wt% (Yield: 85–95 %). Fig. S2 reports an example of the CD analysis.

Sulpho-Cy5.5: Labeling was carried out in double-distilled (dd)H₂O with DMTMM-Cl (0.02 eq.) and sulpho-Cy5.5 amine (0.02 eq.) for 24 h. Purification involved PD10 and Vivaspin® (MWCO 3 kDa) columns. Cy5.5 loading was determined by fluorescence calibration (λ_{ex} = 640 nm, λ_{em} = 700 nm) (Yield: 60–70 %, Efficiency: 70–90 %).

4.2.1.2. Copper-catalyzed crosslinking (StPGA-CL). The synthesis of StPGA-CL via copper-catalyzed azide-alkyne cycloaddition was adapted from Duro-Castaño et al. [18,30] (Fig. S3). Equimolar StPGA-propargyl and StPGA-azide (2 mg/mL each) were mixed in ddH₂O, sonicated, and treated with sodium ascorbate and CuSO₄ under degassed conditions. After 72 h at room temperature, complete conversion (triazole peak at 7.8 ppm, ¹H-NMR) was confirmed. The product was purified via ultrafiltration and Quadrasil® MTU resin to remove copper, yielding a freeze-dried powder (Yield: 70–80 %).

4.2.1.3. Disulfide crosslinking (StPGA-CL-SS). StPGA-CL-SS was synthesized via a two-step process involving the preparation of StPGA-pyridyl disulfide (StPGA-PD) followed by crosslinking through inverse nanoprecipitation, as previously described [67,88]. Briefly, StPGA (100 mg, 1 eq. GAUs) was dissolved in anhydrous DMF (10 mg/mL) and reacted with PD cysteamine (0.15 eq.), using DMTMM-BF₄ (0.15 eq.) as the coupling agent and DIEA for pH adjustment. The reaction was carried out under a nitrogen atmosphere and stirred for 48 h at room temperature. The crude product was precipitated in cold diethyl ether, converted to its sodium salt form, and lyophilized. The resulting StPGA-PD exhibited a conjugation efficiency of 80 % and a yield of 70 %, as determined by ¹H-NMR analysis (Fig. S4A). Crosslinking was achieved

by dissolving StPGA-PD (20 mg) and hexa(ethylene glycol) dithiol (2.5 mg; $M_w = 314$ g/mol) separately in cold ddH₂O, mixing at 4 °C, and immediately precipitating the solution into 400 mL cold acetone under vigorous stirring (900 rpm). After 4 h at room temperature, the reaction was quenched with β -mercaptoethanol (5 eq.). The acetone was evaporated under reduced pressure, and the aqueous phase was purified via dialysis using a 2 kDa MWCO membrane for 48 h against water, followed by lyophilization (Fig. S4B). The successful crosslinking was confirmed by ¹H-NMR comparison of StPGA-PD and StPGA-CL-SS spectra in D₂O and by dynamic light scattering (DLS) analysis of hydrodynamic size (Fig. S4C–D).

4.2.1.4. Odorranalectin (OL) conjugation. N-terminal Conjugation: StPGA (30 mg) was activated with DMTMM-Cl (0.055 eq.) in ddH₂O and reacted with OL (0.05 eq.) (Fig. S5) for 48 h at room temperature, pH 8.0. The product was dialyzed (3.5–5 kDa MWCO), purified via Sephadex® G25, and lyophilized (Yield: 86 %; OL loading: 3.8 mol%) (Fig. S6).

C-terminal Conjugation: OL was Boc-protected using Boc₂O in THF/H₂O, then conjugated to StPGA-Et, previously synthesized via coupling with N-Boc-ethylenediamine (0.12 eq.) in DMF. The final conjugation was done using DMTMM-Cl in MeOH/H₂O for 48 h, followed by Boc-deprotection with TFA, purification, and dialysis (0.5–1 kDa MWCO). Final OL loading was 0.9 mol% (Reaction efficiency: 43 %) (Fig. S7). Figs. S8–S10 reports the characterization of conjugates by NMR and CD.

4.2.1.5. Synthesis of StPGA–HA conjugates. StPGA was functionalized with hexylamine via reaction with N-Boc-1,6-hexanediamine HCl (0.12 eq.) and DMTMM-BF₄ in anhydrous DMF (11.7 mol% loading, 97 % efficiency). After Boc deprotection with TFA, the product was reacted with HA 80 kDa (1 eq.), previously activated with DMTMM-Cl (2.5 eq.), in HEPES buffer (10 mM, pH 8) for 24 h. The conjugate was purified by ultrafiltration (MWCO 100 kDa) and lyophilized. The final StPGA:HA ratio (2:1) was confirmed by ¹H-NMR (peak at 1.88 ppm) (Figs. S11–S14).

4.2.1.6. Synthesis of linear and StPGA–DHA conjugates. LinPGA or StPGA (100 mg) was dissolved in anhydrous DMF and reacted with DHA (0.12 mmol) using DMTMM-BF₄ (0.17 mmol) and DMAP (0.023 mmol) at pH 8.0 under N₂ and protected from light. After 48 h, the product was precipitated in ether, dialyzed (MWCO 3.5–5 kDa) against carbonate buffer (pH 10), and lyophilized. DHA loading ranged from 2 to 6 mol%, depending on backbone type (Figs. S15–S19).

4.2.1.7. Synthesis of hyaluronic–polyglutamate crosslinked hydrogel (HA-CP). HA-CP was prepared via peptidic crosslinking between HA (50 kDa), LinPGA, and L-lysine using DMTMM-Cl as the coupling agent. HA (1 eq.) and LinPGA (0.07 eq.) were mixed, then activated with DMTMM-Cl (0.6 eq. to total carboxyl). L-lysine (0.03 eq.) was added, and pH was adjusted to 7–8. Reaction was stirred overnight, followed by purification via Vivaspin® (MWCO 30 kDa), dialysis, and lyophilization (Yield: 70 %) (Figures S20–S21). Coupling conditions were optimized using LinPGA-Lys-LinPGA as a model (Scheme S1, Table S1), yielding the best results at pH 7–8 and 20 eq. DMTMM to Lys. Crosslinking was confirmed by ¹H-NMR and DOSY-NMR. Scale-up and characterization of HA-CP batches confirmed reproducibility and low impurity content (Figure S22). Additionally, hyaluronidase degradation studies demonstrated greater resistance to HA-CP compared to HA (Figure S23).

4.2.2. Electron microscopy characterization

Electron Microscopy Characterization Transmission electron microscopy (TEM) and Cryogenic TEM (Cryo-TEM) were employed to evaluate the morphology and size of StPGA, StPGA-OL (C-term), and StPGA-CL-SS nanostructures, both alone and embedded within HA-CP matrices

(1–1.33% w/v). Conventional TEM and Cryo-TEM imaging was performed on a T20-TEI Tecnai microscope operated at 200 kV using a LaB6 electron source and a SuperTwin objective lens, providing a point-to-point resolution of 2.4 Å. To enhance contrast, samples were stained with 3% phosphotungstic acid. For Cryo-TEM analysis, vitrified specimens were prepared using a Vitrobot system to preserve the native hydrated state of the nanostructures. The obtained TEM and Cryo-TEM micrographs confirmed the expected nanostructure morphology and their homogeneous distribution within the HA-CP network (Fig. S24).

4.2.3. Ex vivo permeation studies

4.2.3.1. Preparation of sheep nasal mucosa and experimental setup. Freshly excised nasal mucosa was collected from healthy sheep. Samples were kindly donated by the veterinary school at CEU Cardenal Herrera University (Valencia, Spain) under the SANDACH normative and the relevant biosecurity regulations of the University. The superior nasal membrane was identified and separated from the nasal cavity, where turbinates were fully exposed by a longitudinal incision through the nose, and the mucosa was carefully removed from the underlying bone. Maintaining the viability of the excised nasal tissues during the experimental period remains crucial; therefore, the mucosa was carefully removed and immersed in ice-cold phosphate buffer saline (pH 6.4) for transportation to the laboratory within 10 min of animal sacrifice. Mucoadhesion studies were started immediately after mucosal sample excision.

4.2.3.2. Mucosa permeation studies. Fresh sheep mucosa samples were cut into 4 × 4 cm pieces and fixed between the donor and receptor chambers of the Franz diffusion cells (Logan Instruments Corp., USA) so that the stratum corneum was placed with the apical side facing the donor chamber and the basolateral side facing the receiving chamber, resulting in a permeation flow from the apical side to the basolateral side. The receptor chamber was filled with 8 mL PBS (pH 6.4) and mixed with a magnetic stirring bar at 37 °C. Then, 100 μ L 10 mg/mL OG-labeled PGA-based nanocarriers were added to the donor chamber of the Franz cells. In specific cases, HA-CP (Yalic®; 1.33 %w/v) was mixed with the OG-labeled PGA-based platform solution before addition to the donor chamber. Isopropyl alcohol was used as a negative control. Cells were covered with Parafilm™ and aluminum foil to avoid evaporation and light exposure. At established time points up to 24 h, 2 mL aliquots were taken from the receptor chamber, which was immediately refilled with fresh PBS buffer (pH 6.4). We then filtered samples through a Nylon filter (0.22 μ m), and the diffused amount of OG-labeled PGA-based platform was determined spectrophotometrically ($\lambda = 492$ nm) using a Jasco V-550 UV/Vis Spectro-photometer (Tokyo, Japan). Each platform was measured in at least three separate samples. The detector response displayed linearity in a wide concentration range (0.1–10 μ g/mL). OG quantification used two segments of the calibration curve: low concentration range (0.1–0.9 μ g/mL) and high concentration range (0.1–10 μ g/mL). The permeation results represent the mean cumulative values for diffused polymeric formulations at the sampling time point.

4.2.3.3. Nasal-mucosa-on-a-chip (NMoC). The NMoC model represents a customized microfluidic platform with continuous perfusion to provide a steady nutrient supply and efficient metabolic waste removal, specifically designed to support the differentiation and function of human nasal epithelium under ALI conditions. The NMoC model consists of laser-microstructured polymethylmethacrylate (PMMA) layers and a porous polycarbonate (PC; 3 μ m pore size, ipCELLCULTURE™ Track-Etched Membrane Filter Polycarbonate, itip4) membrane. The PC membrane is sandwiched between a PMMA perfusion channel and a microtissue culture well, which hosts the epithelial barrier. The NMoC model is built from bottom to top with a 250 μ m base layer (PMMA), a 250 μ m media layer (PMMA), a 20 μ m membrane (PC), a 1 mm tissue

layer (PMMA), and a 3 mm microtissue well compartment. The different layers are bonded by thermofusion to assemble the NMoC.

4.2.3.3.1. NMoC tissue generation. HNEPCs were isolated from nasal polyps [89] obtained as surgical waste from the University Hospital Tübingen (Germany) (approved by the Ethical Committee of the Eberhard Karls University Tübingen; No. 346/2022BO2). Isolated cells were cultured in Airway Epithelial Cell Growth Medium (AECGM; C-21060; PromoCell), passaged using 0.05 % trypsin-EDTA, and reseeded at a density of 15,000 cells/cm². Prior to seeding, the NMoC model was oxygen plasma-treated to ensure hydrophilicity and sterilization. Following plasma treatment, the platform was flushed with pre-warmed AECGM supplemented with 1 % antibiotic-antimycotic (AA) (15240062, Gibco™). The platform was incubated at 37 °C until cell seeding. Cells were detached from T75 flasks using 0.05 % trypsin-EDTA (4 mL, 5 min, 37 °C), and the enzymatic reaction was stopped with 4 mL trypsin inhibitor (R007100; Gibco™). After centrifugation (300 g, 4 min), the cell suspension was adjusted to 2.5 million cells/mL in AECGM + 1 % AA, and 4 µL cell suspension (10,000 cells) was added to each microtissue well. The platform was incubated (37 °C; 5 % CO₂, 90 % humidity) for 2 h before adding fresh medium. Medium was exchanged daily, and ALI was established on day 5 post-seeding by aspirating the apical medium and initiating perfusion.

Tube tuck connectors (CS-10001764; Darwin Microfluidics) were inserted into the platform's perfusion channel ports to connect the platform to medium perfusion, and the chips were connected to a syringe pump (LA-190, Landgraf Laborsysteme HLL GmbH) via 21 GA stainless steel needles (company, #cat) and Tygon tubing (0.51 mm ID; 100-80 Medical Tubing, Saint-Gobain Performance Plastics Pampus GmbH). Syringes with Luer Lock connectors (4606710V; B. Braun) were used, and the pump was operated in push mode at 20 µL/h. The ALI perfusion condition was maintained for 3 days before subsequent assays.

4.2.3.3.2. Permeation and cytotoxicity assay using the NMoC. Cy5.5-labeled nanocarriers (10 mg/mL) were applied in 2 µL to each microtissue well containing the ALI-cultured nasal epithelium to assess permeation and cytotoxicity. For controls, 0.1 % Triton X-100 in PBS served as a maximal release reference, and PBS alone served as the vehicle control. The NMoC model was perfused at 20 µL/h, and effluent samples were collected hourly to designated timepoints. Cy5.5 fluorescence in 20 µL of collected medium was quantified using a Spark® multimode microplate reader. Cytotoxicity was evaluated by measuring LDH release with the LDH-Glo™ Cytotoxicity Assay (J2380; Promega). Effluent samples were diluted 1:15 in LDH storage buffer (200 mM Tris-HCl (pH 7.3), 10 % Glycerol, 1 % BSA) to preserve enzyme activity during freezing until analysis.

4.2.4. In vivo biodistribution studies

4.2.4.1. Intranasal administration and fluorescence ex vivo monitoring. Mice were randomly divided into representative groups of seven animals, comprising control and treatment groups. Animals were trained the week before the experiment to establish the intranasal administration protocol. The treatment groups received selected candidates (StPGA, StPGA-CL-SS, and StPGA-OL) alone or physically mixed with HA-CP (1.33 %w/v). Before each administration, mice were lightly anesthetized using 3 % isoflurane in oxygen (flow rate: 300 cm³/min). Subsequently, animals were positioned supine with a slightly elevated head to facilitate intranasal administration. All formulations were freshly and aseptically prepared in a biological safety cabinet immediately before dosing. The compounds were dissolved in PBS (pH 7.4) at 30 µg Cy5.5 eq./Kg (n = 7 for each compound). A single dose (20 µL) of the prepared formulation was administered intranasally using a micropipette, with 10 µL delivered into each nostril. The preparations were slowly instilled into the nostrils to allow for inhalation. After 0, 30, 60, 90, 120 min, and 4 h, a representative mouse was euthanized, and blood was collected using heparinized syringes. Major organs (brain, liver,

heart, spleen, kidney, lungs, intestine) were carefully harvested, flushed with 20 mL Dulbecco's phosphate-buffered saline (DPBS), and immediately weighed and subjected to ex vivo fluorescence detection by IVIS® technology (Perkin Elmer Inc., Waltham, MA, USA) with $\lambda_{\text{ex}} = 640$ nm and $\lambda_{\text{em}} = 700$ nm. Two brains were selected from each group for later immunohistochemistry assays and were fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and stored at 4 °C. After 18 h of fixation under orbital agitation, brains were washed with 15 mL DPBS three times and left in 15 mL 30 % sucrose solution in DPBS for 48 h at 4 °C. Finally, brains were washed with 15 mL DPBS three times and conserved in 0.05 % sodium azide solution in DPBS until fixation. The remaining organs were frozen in liquid nitrogen for further ex vivo quantification and stored at −80 °C.

4.2.4.2. Organ homogenization for Cy5.5 fluorescence quantification. The harvested and stored organs at −80 °C were quickly thawed at room temperature and placed on ice. Organs were homogenized in DPBS (pH = 7.4) with an Ultraturrax device using optimized buffer volume, mL/homogenization time, sec/rotation speed, rpm depending on the organ, specifically (mL/sec/rpm): heart (1/60/10000), lungs (1/30/10000), spleen (1/30/10000), liver (2/60/10000), kidney (1.5/60/10000), brain (2/60/7500), intestine (3/60/10000). The Ultraturrax device was washed with ddH₂O and PBS three times and dried after each organ homogenization cycle. The homogenized organ suspension was centrifuged at 4000 rpm for 1 h at 4 °C, and 100 µL supernatant was transferred in triplicate to a black P96 plate for fluorescence measurement with a CLARIOstar® Plus plate reader. The corresponding calibration curve with Cy5.5 in DPBS was prepared in the 0.125–70 ng/mL range, and the fluorescence signal was measured at $\lambda_{\text{ex}} = 640$ nm, $\lambda_{\text{em}} = 700$ nm. Pellets were washed with ddH₂O to assess total conjugate extraction (no significant signal was observed). Considering the amount of polymer injected, the percentage of injected dose (%ID) in each organ was calculated and divided by the organ's weight in grams (%ID/g).

Blood samples were centrifuged (10 min, 4000 rpm, 4 °C) immediately after extraction, plasma (supernatant) was collected, and the fluorescent signal was directly measured (100 µL) in triplicate in a 96-well plate by a reader plate fluorescence detector.

4.2.4.3. Histology of brain distribution. Brains were immersion-fixed in 4 % paraformaldehyde (PFA) and cryoprotected in 30 % sucrose. Subsequently, they were sectioned at 25 µm thickness using a Leica CM1860 UV cryostat. Two series of brain sections were processed differently depending on the type of analysis. One series was used to evaluate Cy5.5 fluorescence and was stained only with 4',6-diamidino-2-phenylindole (DAPI; 1:1,000 in water, 5 min), then mounted directly with FluorSave (Calbiochem-Millipore), without any immunolabeling. A second series was processed for immunofluorescence to identify Cy5.5 fluorescence in specific brain cell types. Endogenous peroxidase activity was blocked using a solution of 10 % methanol and 3 % H₂O₂ in 0.1 M phosphate buffer (PB). Sections were then permeabilized with three washes of 5 min in PTA solution (0.1 % Triton X-100 and 1 mg/mL bovine serum albumin in 0.1 M PBS), followed by incubation overnight at 4 °C with primary antibodies diluted in blocking solution (10 % casein and 5 % normal goat serum in PTA). The following primary antibodies were used to detect astrocytes, neurons, and microglia, respectively: 1:250 mouse anti-GFAP (Millipore, Cat# MAB360, RRID: AB_11212597), 1:100 mouse anti-NeuN (Millipore, Cat# MAB377B, RRID: AB_177621), and 1:500 rabbit anti-Iba1 (FUJIFILM Wako, Cat# 019-19741, RRID: AB_839504). After rinsing in 0.1 M PB, sections were incubated for 1 h at room temperature with secondary antibodies diluted in blocking solution: 1:300 goat anti-mouse Alexa Fluor 488 (Molecular Probes, Cat# A-21424, RRID: AB_141780) and 1:300 goat anti-rabbit Alexa Fluor 555 (Molecular Probes, Cat# A-21428, RRID: AB_141784). Nuclei were counterstained with DAPI, and samples were mounted with FluorSave. Confocal images were acquired using a Leica TCS-SP2-AOBS microscope

and analyzed with FIJI image processing software [90]. Cy5.5-conjugate fluorescence was assessed in five randomly selected fields per region in the hippocampus, layers II/III of the somatomotor cortex, and the olfactory bulb for each mouse ($n = 4$ per time point).

4.3. Statistical analyses

All data are presented as mean $\pm$ SEM and were compared via a two-way ANOVA followed by Šidák's multiple comparisons test using GraphPad Prism version 9.0.0 for Mac OS (GraphPad Software, San Diego, California, USA, www.graphpad.com).

CRedit authorship contribution statement

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Declaration of competing interest

M.J.V. and V.J.N. are inventors of an EU and U.S.-granted patent related to this work filed by CIPF (no. WO2017025298A1 filed 7 August 2015 and published 16 February 2017). D.M. – B, V.J.N, and M.J.V declare that a patent with reference WO2019020344 (A1) has been filed related to the present work. I.C.-S and M.J.V are inventors of an EU-granted patent partly related to this work filed by CIPF, VHIR, UAB, CSIC and ICN2 (PCT/EP2023/083626 29/11/2023, WO2024115612A1). The authors declare that they have no other competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2025.123867>.

Data availability

Data will be made available on request.

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