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Capturing “Extraordinary” Soft-Assembled Charge-Like Polypeptides as a Strategy for Nanocarrier Design


The rational design of nanomedicines is a challenging task given the complex architectures required for the construction of nanosized carriers with embedded therapeutic properties and the complex interface of these materials with the biological environment. Herein, an unexpected charge-like attraction mechanism of self-assembly for star-shaped polyglutamates in nonsalty aqueous solutions is identified, which matches the ubiquitous “ordinary–extraordinary” phenomenon previously described by physicists. For the first time, a bottom-up methodology for the stabilization of these nanosized soft-assembled star-shaped polyglutamates is also described, enabling the translation of theoretical research into nanomaterials with applicability within the drug-delivery field. Covalent capture of these labile assemblies provides access to unprecedented architectures to be used as nanocarriers. The enhanced in vitro and in vivo properties of these novel nanoconstructs as drug-delivery systems highlight the potential of this approach for tumor-localized as well as lymphotropic delivery.

Nanomedicine and nanotechnology have emerged to address unmet medical needs in cancer treatment[1] and recently expanded into other areas including neurological disorders or infectious diseases.[2] The translation of nanoproducts to routine clinical use has demonstrated their potential.[3] A nanocarrier size range between 20 and 100 nm is optimal to enhance blood circulation times and promotes an effective passive targeting by the “enhanced permeability and retention (EPR) effect.”[4] This size range can be achieved by the use of higher molecular weight (MW) biodegradable carriers or the self-assembly of individual unimeric species to yield larger nanostructures. Acquired knowledge in the design of drug delivery systems (DDSs) is raising awareness about the need for fully biodegradable and biocompatible carriers.[5] In this framework, well-defined polypeptides with advanced engineered complexity and structural versatility arise as perfect candidates with proven therapeutic potential and clinical translation.[6] The intrinsic architectural, conformational, and multifunctional properties of branched polypeptides, inaccessible to linear polymers, together with their tunable and feature accelerated manufacture compared to dendrimers, make their application as DDS a potentially exciting proposition.[7]

The rising potential of polymer self-assembled DDS is nowadays only limited to mastering the molecular interactions such as hydrophobic effect, host–guest or electrostatic interactions, among others.[8] While seemingly counterintuitive, charge-like macromolecule aggregation has been described from a theoretical standpoint[9] and represents a ubiquitous phenomenon in biology, involved, for example, in virus capsid and microtubule (F-actin) self-assembly, DNA condensation, and chromosome motion.[10] The successful integration of mean field, counterion condensation, and Flory theories for polyelectrolyte solution macroaggregate has shed light on this self-assembly phenomenon.[11–13] Experimental evidence for charge-like attraction arises from the observation of two diffusional regimes, the “fast” and “slow” modes observed from dynamic light-scattering (DLS) experiments.[13,14] Particularly relevant for the present work is the “slow” mode, also referred as “extraordinary” behavior, where polyions experience attraction at certain concentrations and low salt content to yield aggregated species. Experimental reports of such aggregation mechanism have mainly been reported from scattering techniques and approached from a theoretical standpoint. This may be due to the transient, disordered, and reversible nature of these soft materials, which not only makes sample manipulation and observation difficult, but also hinders their applicability.

In our attempts to develop improved nanosized DDSs based on star-shaped polyglutamates, we identified this unexpected charge-like attraction mechanism of self-assembly. In this study, we used a wide array of techniques to provide consistent...
experimental evidence for the “extraordinary” behavior. For the first time, we show how a bottom-up methodology reliant on the molecular design and covalent stabilization of highly dynamic coassemblies via “extraordinary” behavior allows the construction of otherwise inaccessible nanostructures of 30–100 nm in radius, a size ideal to promote long-blood circulation times and enhanced therapeutic effect, particularly in cancer treatments.

We synthesized a family of well-defined 1,3,5-benzentriacarb-oxamide (BTA)-centered star-shaped polyglutamates (sodium salts) with control on target degree of polymerization, narrow MW distributions, and arm length symmetry in a reproducible and scalable manner (Figure 1a and synthetic details in the Supporting Information).\textsuperscript{[15]} Synthesized compounds differed structurally on the polyglutamate degree of polymerization with 25 or 50 glutamic units per arm and a BTA core motif bearing different spacers (Figure 1a, compounds 1a–e).

To gain a deep understanding of these systems and to acknowledge industrial and clinical translation concerns, we performed a detailed characterization of the compound 1 family. DLS measurements in deionized aqueous solutions for compound 1a revealed nanosized objects with a concentration dependent hydrodynamic radius ($R_h$) in the range of 4–200 nm (Figure 1b, left). The smaller size must be attributed to star-polyglutamate unimers\textsuperscript{[15,16]} whereas the increase in $R_h$ to 40 and 200 nm observed at 1 and 10 mg mL$^{-1}$, respectively, suggested an unexpected self-association of unimers. We also observed a large critical aggregation concentration (CAC) compared to amphiphilic block copolymers, as derived from the scattered intensity versus concentration measurements (CAC $\approx$ 0.5 mg mL$^{-1}$, Figure 1b, middle).\textsuperscript{[17]} Below the CAC, compound 1a was present as an anionic unimeric species with a negative z-potential from $\sim$25 to $\sim$35 mV. Above the CAC, we recorded a sharp increase in the scattered light and the negative z-potential. Of note, the linear counterpart displayed no evidence of this self-assembly behavior at the concentrations studied (Figure S23, Supporting Information).

Derived testis from compound 1a presented CAC values in the same range, independently of the core nature or arm length (Figure S23, Supporting Information). Transmission electron microscopy (TEM) images (also see Figure S24 in the Supporting Information) confirmed the existence of nanosized spherical aggregates of 50–100 and 200–300 nm in size for 1a at 1 mg mL$^{-1}$ (Figure 1c, left) and 10 mg mL$^{-1}$ (Figure 1c, right), respectively, in agreement with DLS data. A closer inspection of morphology evidenced low material density and amorphous nature, as confirmed by the flat X-ray powder diffraction pattern registered for freeze-dried samples of 1a in H$_2$O (Figure S26, Supporting Information).

Although BTA is a widely used self-assembly motif that can induce defined molecular ordering within different supramolecular materials,\textsuperscript{[18]} it requires hydrophilic groups to promote self-assembly in aqueous media.\textsuperscript{[19]} At the molecular level, all attempts to prove the role of BTA in the self-assembly process, performed by fluorescence spectroscopy employing hydrophobic probes (pyrene and Nile Red, Figure S27 and S28, Supporting Information) or small-angle neutron scattering (SANS) contrast experiments of compound 3 (Figure S32, Supporting Information), failed to provide evidence of enriched BTA stack domains.\textsuperscript{[20]} Our findings suggested a poor molecular order in the assemblies and the null participation of the BTA motif in driving the self-association process, as one would expect, since it only represents a small molar fraction of our system. However, circular dichroism (CD) allowed the identification of a characteristic feature for compound 1a, with a recurrent red-shift of the negative band at 200 nm above the CAC (Figure 1d and Figure S34, Supporting Information). This small shift was most likely generated due to partial constraint or orientation of the arms within the assemblies rather than small coil to helix transition of polyglutamate secondary structure upon partial protonation of carboxylic acids, which was negligible at the working pH (Figure S42 and Table S10, Supporting Information). We note that size values did not experience significant changes in a physiologically relevant pH range (from pH 5 to 8) (see Figure S35 in the Supporting Information). In order to disprove the existence of intra- or intermolecular H-bonding along the polypeptidic backbone, we performed DLS titration experiments using common denaturants. Figure 1b (right) demonstrated that while assembly size did not significantly change following the addition of the nonionic denaturant urea, we observed rapid salt-mediated disassembly upon addition of ionic guanidinium chloride at low concentrations ($10 \times 10^{-3}$ M) or salts of opposite nature within the Hofmeister series (Figure S36, Supporting Information).\textsuperscript{[21]} This experiment was key in elucidating the driving forces ruling the polyion
self-assembly observed in our system and suggested ionic interactions as the main actors.

Cryo-TEM imaging for compound 1a confirmed the existence of spherical objects of 30–100 nm in radius (Figure 1f). Visualization of isolated objects permitted the acquisition of tomographic series and 3D reconstruction of the selected nanoparticle (Figure 1g). We noted that the low contrast appearance and the homogeneous low electron density along the aggregate’s structure discounted the existence of a defined core–shell. Furthermore, the irregular surface and globular geometry deviated from ideal spheres (additional 3D tomogram in Figure S25 in the Supporting Information), probably due to the dynamic and loose nature of the assemblies. Analysis of data acquired from SANS experiments for compounds 1a–e in D2O (Figure 1e) demonstrated a best fit for the “Gaussian coil for a Dozier star polymer” model.\textsuperscript{[22]} Fitted parameters revealed a radius of gyration ($R_g$) of 80–100 nm matching the data obtained for compound 1a via DLS and electron microscopy and again suggested the self-association of unimeric species. In the absence of interparticle interactions (where the interparticle structure factor value is $S(Q) \approx 1$, see Figure S29 in the Supporting Information for more details), the Flory component value variation ($\xi$) was found to be small (close to $\frac{1}{2}$), indicating a relatively poor solvent and suggested that the studied polymers were in a collapsed conformation (dense stars). Furthermore, packing close to the core was influenced by the packing efficiency of the substituents (i.e., the polymer packed around a larger overall excluded volume for ethyl than hexyl). Moving toward the outer layers, it seemed that the arms tended to reorganize themselves around a fixed core; hence, the core is simply more “wrapped” as MW increases, allowing the $R_g$ to remain relatively unchanged within the assemblies. The $\xi$ parameter provides an indication of how the polymer swelling changes upon the distance from the core. The $\xi$ parameters were clearly dependent on the substituent used, and varied in the order: ethyl (1a,b) > 3,6-dioxa-8-octane (DOOA) (1c,d) > hexyl (1d).

When combined with the Flory parameter, this provided a consistent picture of how the conformation varies with MW: for larger $R_g$ values the larger core area to cover results in more swollen outer layers and higher $\xi$ values. $R_g$ values varied in the same order as $\xi$: ethyl > DOOA > hexyl substituents in the BTA core; but, surprisingly, there was no dependence on MW (also see Figure 1e and Figure S29 in the Supporting Information), a key concept for the 3D shape elucidation (Figure 1h).

In summary, aggregation of star-shaped polyglutamates occurred in a nonsalty aqueous environment following polyion concentration dependence with a defined and large CAC together with a fast disassembly upon increasing ionic strength. Lack of spectroscopic features points toward a rather amorphous material with only a recurring characteristic signature involving partial constrains of polyglutamate chains. More importantly, evidence suggested that the self-assembly phenomena proceeded via ionic interactions. These features are characteristic fingerprints of the ubiquitous “ordinary to extraordinary” behavior transition in the dynamics of charged macromolecules in aqueous solutions.\textsuperscript{[11–13]} Although the rational has been controversial for years, there is now a consensus to rationalize the self-assembly of charge-like polyions based on counterion condensation theory. This theory states that electrostatic potential becomes attractive as the modeled lines of charges approach within the Debye length and the condensed counterions are merged in a shared cloud.\textsuperscript{[12]} Recently, Muthukumar has approached this phenomena collecting reported data over the last decades.\textsuperscript{[13]} Very interestingly, this model predicts a threshold value of a combination of polyion (CAC) and electrolyte concentration (millimolar range) for the transition to the “extraordinary” behavior. Under these conditions, the observed “slow” diffusional mode results from the presence of polyion clusters due to the formation of transient dipoles arising from adsorbed counterions on polyion backbone that can act as physical cross-linking points. This rational is in agreement with the observed behavior on compounds 1a–e, which displayed a CAC threshold and electrolyte concentration for the self-assembly within the range predicted by Muthukumar. The redshift observed in CD analysis can also be linked to the formation of physical cross-linking dipoles. The fact that we did not observe this behavior for the linear counterparts within the concentrations studied points to the influence of branching, topology, and charge density of compounds 1 that would promote the formation of these cross-linking dipoles.

When compared to reported self-assembled DDS, the architectures generated via “extraordinary” behavior inherently display differential physicochemical features that mediate interactions with biological systems, including size, surface hydrophilicity, charge, lack of internal structure, and enhanced deformability.\textsuperscript{[23]} To assess the biological performance of our new architectures, we aimed to covalently capture our assemblies, as the loose nature of uncaptured assemblies will result in rapid disaggregation in biological media due to increased ionic strength ($>130 \times 10^{-3} \text{m}$) and large dilution rates.\textsuperscript{[34]} Toward this aim, we studied the dynamics, reversibility, and coassembly properties of these systems via stochastic optical reconstruction microscopy (STORM), Förster resonance energy transfer (FRET),\textsuperscript{[35]} and nuclear magnetic resonance (NMR) techniques.

We modified compound 1a on side chains following well-established methodologies\textsuperscript{[26]} with Cy3 (compound 2c) and Cy5 (compound 2d), a FRET pair suitable for STORM microscopy\textsuperscript{[25,27]} (Figure 2a and Figure S15–S18 and Table S3 in the Supporting Information). We obtained a concentration-dependent FRET signal when compounds 2c and 2d were coassembled, indicating that the two dyes (and therefore the two unimers) were in close proximity within common aggregates (Figure 2b). A key feature of supramolecular aggregates is their dynamic nature, i.e., their ability to continuously assemble and disassemble exchanging monomers. We studied monomer exchange kinetics between preassembled compounds 2c and 2d by FRET. Immediately after mixing, a plateau in the signal ratio (and therefore, an equilibrium state) occurred before the first observation time, highlighting the very dynamic nature of our systems.\textsuperscript{[27]} To confirm these indirect spectroscopic observations, we also performed super-resolution STORM experiments for the 2c and 2d compounds. Figure 2d demonstrates that all aggregates were single-colored in single component samples (Figure 2d, left); however, both unimers were present within the same nanoassemblies after 2 min of mixing, confirming the rapid monomer exchange observed in FRET studies. Notably, coassembled aggregates of 100–200 nm coexisted with smaller single colored structures, likely to be unimers or low-order
oligomers (Figure 2e). Overall, these experiments proved the highly dynamic and reversible nature of the assemblies, evidencing the possibility to build coassembled multifunctional DDS and also stressing the need of stabilization for application in biological environments.

To this end, we modified compound 1a at the side chain with different degrees of functionalization of azides (compound 2a) or alkynes (compound 2b) pursuing a nonreversible covalent stabilization of the assemblies through copper-catalyzed alkyn/azide cycloaddition (CuAAC) (Figure 2a and Figure S12–S14 and Table S2 (Supporting Information)). Although functionalization did not significantly alter the assemblies (up to a 30% molar glutamic acid units substitution, as evidenced by DLS, Figure S37–S40 and Table S8 and S9, Supporting Information), the modification rates for the covalent capture were established as 5% and 10% for compounds 2a and 2b, respectively, displaying sizes similar to model compound 1a (Figure S30 and S31 and Table S6 and S7, Supporting Information).

We next confirmed the coassembly of compounds 2a and 2b by pulsed-field-gradient spin-echo (PFGSE) NMR experiments[28] and determined diffusion coefficients of compounds 1a, 2a, and 2b in D2O above (2 mg mL−1) and below (0.1 mg mL−1) CAC values (Figure 3). Diffusion coefficients in the unimeric state (3 × 10−11 m2 s−1) were found to be one order of magnitude higher than those for aggregated nanoassemblies (4.5 × 10−12 m2 s−1), indicating R<sub>0</sub> values of 6 and 44 nm for unimers and assemblies, respectively, in agreement with DLS data (Figure 3b and Figure S43–S45 (Supporting Information)). We doped a sample containing preassembled compound 2b with 2a at a concentration below its CAC. As shown in Figure 3b, the incorporation of 2a unimers within 2b nanoassemblies...
was evidenced by the $D_f$ value obtained for 2a corresponding to self-assembled 2b ($\approx 5 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$). We obtained direct proof for spatial proximity through nuclear Overhauser effect spectroscopy (NOESY) NMR experiments where we identified a clear correlation between azide and alkyne motifs (2a and 2b, respectively) (Figure S46, Supporting Information).

Our results point to the feasibility of a covalent capture strategy using CuAAC (Scheme S4 and Figure S19, Supporting Information) and we next monitored assembly stability during coupling conditions (ionic strength $= 2 \times 10^{-3}$ m) of a 1:1 mixture ratio of compounds 2a and 2b via DLS (Figure 3d, left). Results showed that after CuAAC reaction, the self-assembled polyglutamates were stable following dilution (with a slight compaction to $R_h$ of 30–40 nm also evidenced by cryo-TEM image shown in Figure 3c) or an increased presence of ionic species. This stability differed from the results found for a physical mixture as revealed by DLS (Figure 3d) and SANS experiments (Figure S31 and Table S7, Supporting Information). With regard to industrial and clinical concerns, we found the assembly mechanism and covalent capture strategy to be highly robust and reproducible (Figure S37–S41 and Table S8 and S9, Supporting Information). The covalent capture of dynamic assemblies is a highly attractive proposition, as they have the potential to be applied in novel DDS strategies containing unique features that could yield advantageous biological consequences.

We labeled compound 4 with fluorescence dyes to monitor in vitro and in vivo performance (Figure 4), yielding compounds 5a (bearing Oregon Green, for live-cell imaging, see the Supporting Information) and 5b (with Cy5.5, for in vivo imaging). Importantly, surface modification of compound 4 did not induce structural changes in the parental structure (Figure 4a,b and Figure S34 and S38 and Table S8 and S9,
Figure 4. Biological validation of the covalently captured nanostructures as drug delivery systems. a) Schematic representation of the surface modification of compound 4 with fluorescence dyes. b) Nanoparticle Tracking Analysis (NTA) in ddH$_2$O shows particle distribution of compound 5b in agreement with DLS and Cryo-TEM. c) SH-SY5Y cell viability by MTS assay in response to compound 4 (72 h) demonstrated lack of toxicity ($n > 3$, mean ± SEM). d) Cell-associated
(Supporting Information). Cell viability assays using SH-SY5Y neuroblastoma cells as model cancer cell line and human fibroblasts derived from new-born foreskin as a normal cell model revealed absence of cell toxicity up to the concentrations tested, (see Figure S48 in the Supporting Information), suggesting the feasibility of the CuAAC strategy after proper Cu removal (Figure 4c). A lack of toxic side effects was also demonstrated via histological analysis of tissue slices of key organs extracted from 5b-treated animals at 24 and 72 h postinjection (Figure S53, Supporting Information).

As expected for macromolecular systems of such origin,[29] an energy-dependent uptake was observed (Figure 4d) with a clear co-localization of compound 5a with LysoTracker Red indicating the suitability of these nanocarriers for lysosomotropic drug delivery.[30] (Figure 4e). Importantly, in neuroblastoma cells, a significantly enhanced in vitro cell internalization rate for compound 5a when compared with linear-polyglutamate and star unimer (1a), was demonstrated by flow cytometry and live-cell confocal microscopy, probably due to the larger size and greater negative z-potential (Figure S49 and S50, Supporting Information).[31] In vivo biodistribution of 5b in healthy mice revealed renal excretion profiles, as observed for other polyglutamates.[31] Larger accumulation in all organs generally occurred for 5b and, importantly, we also observed significantly greater terminal and accumulation half-life for 5b (t1/2 beta 115.6 h and t1/2 alpha 1.1 h) when compared to the linear (0.6 and 0.10 h) or star unimer (1a) counterparts (12 and 0.15 h), respectively (Figure S51 and S52 and Table S11, Supporting Information).[31] Increased half-life may enhance passive targeting by the EPR effect when used in well-vascularized objects in the range of 30–100 nm in radius, a highly negative z-potential with a highly dynamic equilibrium, and a rapid disassembly upon an effective charge screening. This self-assembly mechanism was found to be driven by ionic interactions following a recently reported model, where the adsorbed counterions are shared in a merged cloud creating transient dipoles in the polyanionic backbone that can act as cross-linking points. We constructed these biodegradable and biocompatible self-assembled cross-linked star-shaped polyglutamates using a reproducible and scalable bottom-up methodology followed by a covalent stabilization. This strategy enabled in vitro cell internalization rate, and significantly greater terminal and accumulation half-life in vivo. These results allow us to envisage these systems as promising nanocarriers for therapeutic or diagnostic applications, especially in anticancer treatments. Additionally, further studies to identify the mechanism for the significant accumulation found in the lymph nodes will open up a wide range of opportunities for the currently unsuccessful clinical approaches to target lymph node metastasis, imaging of sentinel lymph nodes, and cancer immunotherapy.

**Experimental Section**

**Synthesis of Star-Shaped Poly(γ-glutamates):** Detailed synthetic methodologies and full characterization of the initiators, monomers, polymerizations, deprotection steps, postpolymerization modifications, and covalent capture strategies are fully described in the Section “Synthetic Procedures” in the Supporting information. DLS: DLS measurements were performed using a Malvern ZetasizerNanoZS instrument, equipped with a 532 nm laser at a fixed scattering angle of 173°. Solutions were sonicated for 10 min and

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fluorescence (CAF) over time of the energy-dependent uptake (experiment at 37–4 °C to exclude binding) shows a significant increase for compound 5a (n ≥ 3, mean ± SEM). e) Confocal image of compound 5a uptake at 2 h post-treatment in SH-SY5Y cells following a pulse–chase experiment (Blue-Hoechst 33342 for nuclei; Green-Oregon-green for compound 5a; Red-LysoTracker Red). Colocalization with LysoTracker marker was observed (yellow). f) In vivo biodistribution after intravenous injection of compound 5b shows renal excretion profiles with accumulation in lymph nodes. Data expressed as normalized % injected dose (ID) per gram of tissue (n ≥ 5, mean ± SEM). g) Pharmacokinetic parameters obtained from compound 5b plasmatic profiles. h) In vivo biodistribution of compound 5b toward 1a and linear PGA at 1 and 4 h postinjection confirms the clear significantly higher accumulation of 5b in spleen and lymph nodes (axillary, ALN and cervical, CLN). Data expressed as normalized % injected dose (ID) per gram of tissue (n ≥ 5, mean ± standard error of the mean (SEM)).
allowed to age for the required time, filtered through a 0.45 μm cellulose membrane filter, and measured. Size distribution was measured (radius, nm) for each polymer in triplicate with n > 3 measurements.

For size measurements, polymer solutions were prepared under different conditions (double-deionized water, ddH₂O, phosphate buffers, or salt containing solutions at different concentrations and temperatures). Automatic optimization of beam focusing and attenuation was applied for each sample. CAC was determined using a previously described method.⁴³⁷ TEM Images: TEM images were recorded using a JEOL 2100 transmission electron microscope. Samples of polyglutamates were applied directly onto carbon film on 200 mesh copper grids. Excess of sample was carefully removed by capillarity, and the grids were immediately stained with one drop of 0.1% phosphotungstic acid for the 3 excess chains achieved by capillary action.

Cryo-TEM Tomography: In order to achieve the 3D characterization of star-shaped self-assembled systems, a series of different electron tomograms was obtained. The sample was incubated in Quantifoil R3.5/1 holey carbon copper grids and vitrified using standard procedures with a Vitrobot (FEI). The samples included 10 nm gold nanoparticles (as fiducial markers) used in alignment process. Vitrified grids were transferred into a 914 high tilt cryo transfer tomography holder (Gatan Inc., USA). Samples were transferred into a 914 high tilt cryo transfer tomography holder (Gatan Inc., Karlsruhe, Germany). Samples were prepared at the desired temperature, and temperatures). Automatic optimization of beam focusing and attenuation was applied for each sample. CAC was determined using a previously described method.⁴³⁷ TEM Images: TEM images were recorded using a JEOL 2100 transmission electron microscope. Samples of polyglutamates were applied directly onto carbon film on 200 mesh copper grids. Excess of sample was carefully removed by capillarity, and the grids were immediately stained with one drop of 0.1% phosphotungstic acid for the 3 excess chains achieved by capillary action.

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each were acquired with the strength of the diffusion gradient varying between 5% and 95%. The lengths of the diffusion gradient and the stimulated echo were optimized for each sample. Typical values were 
\[\delta = 1.5 \text{ ms} \] for the analysis of nonaggregated species (0.1 mg mL\(^{-1}\)) and 5 ms to study the nanospecies. 

**NOE Experiments (500 MHz):** 2D NOE experiments (NOESY) where recorded with a mixing time of 100 ms with 128 scans. 

**Cell-Culture Protocols:** SHSY5Y cells from ATCC were cultured in Dulbecco’s modified Eagle medium (DMEM) media supplemented with fetal bovine serum. Cells were maintained at 37 °C in an atmosphere of 5% carbon dioxide and 95% air. Medium was replaced every 2–3 d and underwent passage once weekly when 80% of cell confluence was reached. 

**Assay for Cell Viability:** Cells were seeded in sterile 96-well microtiter plates at a cell density of 35,000 cells cm\(^{-2}\) for SHSY5Y. Plates were incubated for 24 h and compounds (0.2 µm filter sterilized) were then added to give a final concentration of 0–3 mg mL\(^{-1}\). After 72 h of incubation, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium) (MTS)/phenazine methosulfate (PMS) (20:1) (10 µL of manufacturer solution) was added to each well, and the cells were incubated for a further 2 h. Optical density of each well was measured at 490 nm spectrophotometrically using Victor\(^\text{\textregistered}\)-Wallac plate reader. The absorbance values were measured at each time point and uptake of cell viability taken as 100% cell viability of untreated control cells. 

**Cellular Uptake by Flow Cytometry of Oregon Green-Labeled Polymers in SHSY5Y Cells:** SHSY5Y cells were seeded in 6-well plates at a density of 35,000 cells cm\(^{-2}\) (1 mL cell suspension per well) and allowed to adhere for 24 h. In binding experiments conducted at 4 °C, cells were preincubated at this temperature for 30 min prior to starting the experiment. For both experiments, 4 and 37 °C, the cathepsin B inhibitor CA-074 (0.4 µL from a solution of 5 × 10\(^{-6}\) M to reach a final concentration of 2 × 10\(^{-6}\) M) was added 30 min before the addition of the conjugate. Then, 10 µL of Oregon Green (OG)-labeled polymer (0.01 mg OG per mL) was added at different time points from 0 to 300 min while cells were incubated either at 37 or 4 °C for each experiment. Finally, cells were placed on ice to inhibit energy dependent mechanisms and washed twice with cold PBS–BSA 0.1%. (PBS supplemented with bovine serum albumin (BSA)). Then, cells were resuspended in 0.5 mL of cold PBS using a cell scraper. Finally, the cell pellet was placed in flow cytometer tubes. Cell-associated fluorescence (CAF) was then analyzed using a Becton Dickinson fluorescence-activated cell sorting (FACS)Calibur cytometer (California, USA) equipped with an argon laser (488 nm) and emission filter for 550 nm. Data collection involved 10,000 counts per event and the organ, a calibration curve was previously performed. 

**Confocal Fluorescence Microscopy—Live-Cell Imaging:** For live-cell imaging, SHSY5Y cells were seeded at a density of 35,000 cells cm\(^{-2}\), on glass bottom culture dishes (1 cm\(^2\) Petri plate) and allowed to adhere for 24 h at 37 °C. The experiment was performed following a pulse–chase mode at 37 °C. First, the cathepsin B inhibitor CA-074 (0.4 µL from a solution of 5 × 10\(^{-6}\) M to reach a final concentration of 2 × 10\(^{-6}\) M) was added 30 min prior the addition of the St–PGA–OG. Then, 10 µL of OG-labeled polymer (0.01 mg OG per mL) was added and the cells were incubated for 2 h at 37 °C (pulse). The medium was removed and cells were washed twice with PBS. 1 mL of medium containing 2 × 10\(^{-6}\) M of the CA-074 inhibitor was then added, and cells were incubated for 4 h at 37 °C (chase). 30 min before washing the cells with PBS–BSA 0.1%, the nuclear marker Hoechst (1 µL from a solution of 5 × 10\(^{-5}\) M) and the lysosomal marker LysoTracker Red (0.75 µL from a solution of 100 × 10\(^{-6}\) M) were added and incubated for 20 min in order to identify possible co-localizations and therefore establish an endocytic pathway. Finally, cells were washed with PBS–BSA 0.1%. Then, the glass was removed and placed on the microscope chamber with fresh media containing 2 × 10\(^{-5}\) M of CA-074 inhibitor. Samples were analyzed under the microscope. Images were captured with an inverted DM IRE2 microscope equipped with a λ-blue 60X oil immersion objective and handled with a C9GE2 SP2 system, equipped with an acoustic optical beam splitter. Excitation was performed with an argon laser (OG 496 nm), HeNe laser (LysoTracker Red 594 nm), and blue diode (Hoechst 405 nm). Images were captured at an 8-bit gray scale and processed with Leica Confocal Software (LCS) (version 2.1.1347a, Leica Germany) containing multicolor, macro-, and 3D components. Control cells that followed the same incubation time were also analyzed to establish the autofluorescence, as well as cells treated only with Hoechst or LysoTracker Red. 

**Biodistribution Experiment and Fluorescence Quantification:** Biodistribution experiments were carried out using female athymic nude mice of five to seven weeks from Envigo. Animals were housed on a 12 h light and 12 h dark cycle. Water and food was provided ad libitum during the whole experiments in all cases, and general aspect, behavior, and body weight were evaluated daily to ensure animal wellness. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Centro de Investigación Príncipe Felipe (Valencia, Spain). For the pharmacokinetics and biodistribution study, 30 mice were used, with 25 ± 4.5 mg of polymer dosing (0.1 mg mL\(^{-1}\)) of the CA-074 inhibitor polymer was administered intravenously through the tail vein at a dose of 4.15 mg kg\(^{-1}\) Cy5.5 eq. (5.41 mg mL\(^{-1}\) polymer) in saline. Mice were euthanized at 0, 4, 8, 24, 48, and 72 h postadministration. Blood, major organs (heart, lungs, spleen, liver, kidneys, and brain), and small organs (ovaries, adrenal glands, axillary and cervical lymph nodes) were harvested after flushing with 10 mL PBS. Then, organs were weighted and stored at −80 °C for subsequent homogenization and fluorescence quantification. Blood samples were centrifuged immediately after extraction (10 min, 4000 rpm, 4 °C) and plasma (supernatant) and pellet were stored separately at −80 °C. For fluorescence quantification, major organs and fallopian tube were homogenized in Dulbecco’s phosphate buffered-saline (DPBS) pH = 7.4 at a specific concentration by means of Ultraturrax device and the rest of the small organs were homogenized manually with a potter glass (see Table S5 in the Supporting Information). Suspensions obtained were centrifuged (4000 rpm, 1 h, 4 °C) and supernatants were collected for fluorescence measurement. Supernatants of organs collected and plasma were measured (100 µL) per triplicate in 96 well-plates using Victor\(^\text{\textregistered}\)-Wallace by fluorescence (595 nm excitation and 680 nm emission). To relate the fluorescence with the concentration of conjugate present in the organ, a calibration curve was previously performed. 

**Statistical Analysis:** Data from the experiments were analyzed using one-way ANOVA and Bonferroni posthoc for pairwise comparison. In all cases, differences were considered to be significant when **p < 0.001; ***p < 0.001; *p < 0.05; ns: nonsignificant. **p < 0.001 5b versus star, ##p < 0.001 5b versus PCA, t tp < 0.001 5b versus Cy5.5.** 

**Pharmacokinetics:** The data analysis was performed by nonlinear regression using the SOLVER function of the spreadsheet program Microsoft Excel as has been described by Martin et al.[2] (the pharmacokinetic parameters were A, B, ALPHA, and BETA). From these parameters, several derived pharmacokinetic parameters were computed: AUC (A/ALPHA + B/BETA), CL (D/AUC), Vss, Cmax (A + B), and apparent terminal half-life. As plasma concentrations often span a wide range, it is useful to employ a weighting procedure for the raw data that allows one to fit low concentrations and high concentrations simultaneously. Weighting: 1/Y\(^2\). 

**Histology:** Key organs for the determination of the absence of toxicity after 5b administration to mice were extracted from euthanized animals at 24 and 72 h biodistribution time points. After being washed with PBS, kidneys, livers, and spleen were fixed in paraformaldehyde (PFA) for 24 h. Then, the excess of PFA was removed by washing with PBS under strong agitation (200 rpm) for 20 min, five times. Finally, samples were stored in a solution of PBS with 0.05% of sodium azide. In order to include the sample in paraffin, a previous dehydation of the sample through 2 min incubation in increased degree of alcohol solutions (30%, 50%, 70%, 96%, and 99.9%) was performed, followed by two xylol washes of 1 min
to finally include the sample in paraffin. Then, the paraffin block was cut in 5 μm slide and set up in SuperFrost plus glass slide to hematoxylin–
eosin staining. For hematoxylin–eosin staining, previous tissue slides were
dereparaffinized with xylene and then rehydrated with a decreasing
battery of ethanol solutions (99.9%, 96%, 70%) and water (5 min)
followed by Dako hematoxylin staining for 2.5 min. Tissue samples were
then washed with deionized water (1.5 min bath), blueing buffer (1 min),
and water (1.5 min). Then, tissue slides were incubated 2 min in lithium
bicarbonate and HCl 0.25% in ethanol 70% in order remove the excess of
hematoxylin staining. Following that, tissue slides were incubated with
Dakocytion for 2 min. Finally, dehydration was performed by washing
with 96% ethanol (30 s) and 99.9% ethanol (2.5 min). The slide was
then mounted with Eukitt.

Supporting Information
Supporting Information is available from the Wiley Online Library or
from the author.

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Conflict of Interest
ADC, V.J.N, and MJV declare that a patent with reference
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