

Cyclodextrin-Based Nanogels with Chemistry-Tunable Intracellular Stability and Distribution

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Introduction: Cyclodextrin-based nanogels (CD-nGels) uniquely enable encapsulation of hydrophobic pharmaceuticals within hydrophilic networks through host–guest interactions, thereby improving drug solubility, stability, and therapeutic efficacy.

Methods: Here, we report the surfactant-free synthesis of Nile Blue-labeled fluorescent CD-nGels with tunable hydrolytic properties by integrating amide or ester linkages in both β -cyclodextrin (β CD) moieties and crosslinking sites. Three formulations were prepared with progressively increasing hydrolytic sensitivity: NG1 (fully amide-linked), NG2 (ester-functionalized β CD with amide cross-linking), and NG3 (ester linkages in both β CD and crosslinker domains).

Results: The resulting CD-nGels were monodisperse with hydrodynamic diameters ranging from 247 to 431 nm. Hydrolysis study in mildly acidic conditions (pH 5.1) and in intracellular-mimicking MCF-7 lysate demonstrated that the structural stability is predominantly dictated by chemical compositions. Coumarin-6 (C6), a hydrophobic fluorescent model drug, was efficiently encapsulated via host-guest interactions to visualize the intracellular redistribution after cellular uptake. The confocal microscopy revealed that the three CD-nGel formulations exhibited progressively enhanced intracellular degradability, leading to distinct intracellular fluorescence distributions: while the fully amide-linked NG1 maintained a compact intracellular fluorescence pattern, the ester-containing NG2 and NG3 exhibited progressively diffuse cytoplasmic signals consistent with reduced structural stability.

Discussion: Overall, this modular platform enables chemical tuning of intracellular stability and distribution of CD-nGels, providing a design basis for the future development of CD-based nGels for controlled intracellular drug delivery applications.

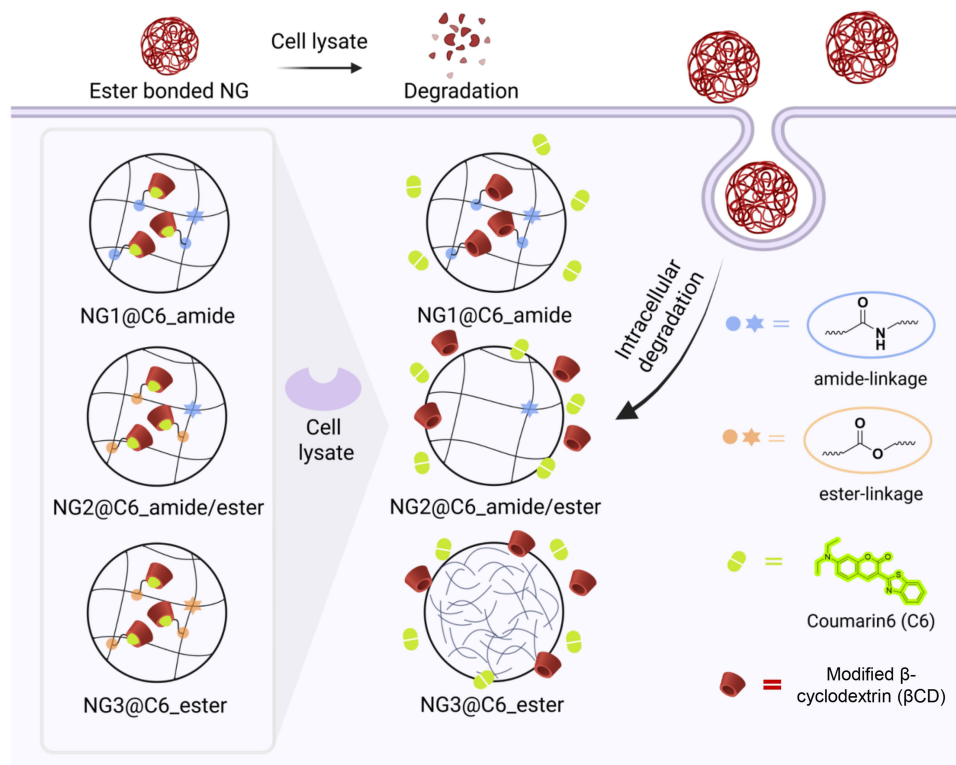
Keywords: cyclodextrin-based nanogels, hydrolytic stability, host–guest interactions, intracellular distribution

Introduction

In addition to conventional small-molecular drugs, emerging pharmaceutical agents such as proteins, peptides, nucleic acids, functionalized antibiotics, and synthetic nanomedicines have significantly expanded the therapeutic landscape.^{1–5} However, these advances also introduce new challenges, including poor aqueous solubility, instability, drug toxicity, limited efficacy, and off-targeting possibility.^{6–8} In particular, intracellular delivery remains a bottleneck for various medical applications, especially for chemotherapeutic treatments, which require precise release to achieve therapeutic efficacy.^{9,10} Therefore, growing efforts have been dedicated to developing advanced drug delivery systems and intracellular delivery strategies to address these limitations and enhance therapeutic performance.^{11–14}

Nanostructured delivery systems have attracted significant interest due to their enhanced cellular interactions and improved internalization with respect to the free drug.^{11,15} Various nanocarriers, including polymeric, lipid-based, and inorganic nanoparticles (NPs), have been developed to encapsulate therapeutic agents and improve drug efficacy.^{16–21} However, many of these systems still face critical limitations. For instance, liposomes are widely used due to their biocompatibility and drug-loading capacity, but they suffer from poor stability during storage, as well as challenges in

Graphical Abstract



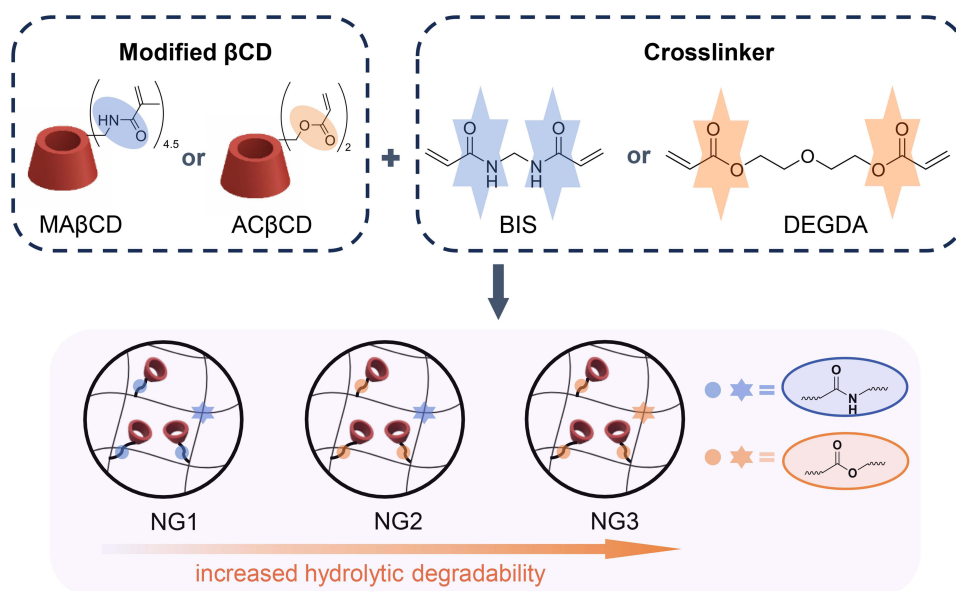
large-scale production.²² Similarly, inorganic NPs such as gold NPs and iron oxide NPs offer unique optical and thermal properties but often lack biodegradability and may accumulate in tissues, raising concerns about long-term toxicity.^{19,23} To circumvent these accumulations, it is therefore advantageous to use structures that are able to degrade such as biodegradable polymeric NPs composed of polylactide or polyglycolide, and inorganic mesoporous silica NPs.^{24,25} For instance, Li's group investigated a novel mesoporous silica NP system with good biocompatibility and low toxicity, which can be applied as a drug delivery platform. The resulting NPs were shown to degrade completely under simulated physiological conditions over a period of 2–13 weeks, with the degradation rate governed by their wettability.²⁶ Even though these particles serve their purpose and are able to deliver various kinds of pharmaceutically active agents, these particles are stiff and recent studies showed that stiffer particles are not accumulating everywhere that effectively, such as in the brain, and are more easily cleared by phagocytosis and having a reduced circulation time within the body.^{27–30} Hence, softer particles offer tremendous opportunity to complement the drug delivery field of which nanogels (nGels) provide excellent possibilities.^{16,31}

The hydrogel-based NPs, nGels, are nanosized soft colloidal particles composed of three-dimensional (3D) water-swollen polymeric networks.³² Owing to their high surface-to-volume ratio, tunable responsiveness, facile surface modification, and low cytotoxicity, nGels have shown great promise as advanced drug delivery systems.^{11,15,33} In particular, their hydrophilic interior allows them to disperse easily in aqueous environments, forming free-flowing solutions that are suitable for administration in liquid dosage forms.^{34,35} However, loading hydrophobic drugs into hydrophilic nGels remains a challenge. This incompatibility can lead to low encapsulation efficiency and may even trigger the shrinkage of nGels due to phase separation.^{6,36} Recent studies have focused on integrating complexation agents into nGel structures to enhance drug loading capacity and enable controlled release while improving overall bioavailability.^{37,38} Cyclodextrin (CD) is a macrocyclic oligosaccharide composed of six to eight glucose subunits linked

by 1,4-glycosidic bonds, typically produced through enzymatic hydrolysis of starch.^{39,40} Owing to its biocompatibility, cost-effectiveness, and pharmacological inertness, CD has been widely used as a pharmaceutical excipient in various drug delivery systems.^{32,41} Notably, although the outer surface of CD is hydrophilic, its inner cavity is relatively hydrophobic. This unique structure enables CD to form host-guest inclusion complexes (ICs) with small hydrophobic drug molecules and thereby offers a promising strategy to enhance drug loading without changing the overall hydrophilicity of the gel network.^{39,42,43}

However, one of the major limitations of CDs in pharmaceutical applications is the difficulty in releasing the drug from the CD cavity under physiological conditions.³² While weakly or moderately bound drugs may dissociate upon dilution in vivo, strongly bound compounds (eg, binding constants $\geq 10^4 \text{ M}^{-1}$) often require competitive displacement by plasma or tissue components to be released effectively.⁴⁴ To achieve reliable and efficient drug release, increasing attention has been directed toward designing carriers that respond to specific biological stimuli.^{45–47} In particular, the acidic tumor microenvironment (pH 6.5–6.8) and intracellular organelles such as endosomes and lysosomes (pH 4.0–6.0) offer available pH gradients relative to normal tissues (pH 7.4).^{48,49} Accordingly, pH-sensitive nGels have been engineered to enable intracellular drug release based on these environmental differences.^{48,50} In addition to pH, intracellular enzymes such as esterases, abundantly expressed in many cell types, can serve as biological triggers for controlled drug release.^{51,52} In this case, ester linkages are particularly attractive due to their susceptibility to both acidic hydrolysis and enzymatic cleavage.^{53–55} Under mildly acidic conditions, protonation of the carbonyl group increases its electrophilicity, facilitating nucleophilic attack by water and leading to cleavage of the ester bond into the corresponding carboxylic acid and alcohol products.^{56–58} After cellular uptake, this process may be further promoted in enzyme-rich intracellular microenvironments, particularly along the acidic endo-lysosomal pathway.^{51,59,60} Notably, although ester linkages therefore provide convenient handles for introducing degradability, their hydrolysis can generate acidic byproducts (eg, acrylic acid from acrylate esters), which may lower the local pH and potentially induce concentration-dependent cytotoxic effects.^{56,61} Consequently, cytocompatibility evaluation is necessary before in vivo applications. In our previous work, we demonstrated that the intrinsic difference in reactivity between ester and amide linkages can be utilized to tune nGel degradability in intracellular environments.⁵² While amide-linked nGels remained stable both inside and outside cells, those containing acrylic ester bonds underwent intracellular degradation. However, degradable nGels are most commonly designed by introducing labile polymeric backbones or crosslinkers (eg, acid-labile ketals, redox-labile disulfides, or photolabile linkers) that cleave under specific conditions.^{52,62–64} CD units are typically incorporated to mainly provide host–guest binding sites for hydrophobic pharmaceuticals.^{32,65,66} Approaches that simultaneously modulate hydrolytic stability at both the CD host units and the crosslinking networks has been explored far less. To bridge this gap, a series of CD-nGel in which degradability is programmable via the chemical design of both CD moieties and crosslinking domains has been designed. While the cleavage of ester bonds within crosslinking domains facilitates nGel erosion by directly reducing network connectivity, ester linkages associated with CD units offer an additional handle for fine-tuning intracellular degradation and distribution.

Herein, we designed a series of CD-nGels using two types of modified CDs: amide-linked methacrylated amino- β -cyclodextrin (MA β CD) and ester-linked acrylated β -cyclodextrin (AC β CD). Two crosslinkers with different hydrolytic sensitivities were employed: the amide-based N,N'-methylene bis(acrylamide) (BIS) and the ester-based diethylene glycol diacrylate (DEGDA). Combining these CD monomers and crosslinkers yielded three CD-nGel formulations (NG1, NG2, and NG3) with progressively enhanced hydrolytic degradability (Scheme 1). We aimed to investigate how these specific chemical linkages within both CD units and crosslinking domains influences the intracellular stability and distribution of nGels following cellular uptake. Hydrolysis behaviors were systematically evaluated under mildly acidic conditions (pH 5.1) and in intracellular- mimicking Michigan Cancer Foundation-7 (MCF-7) human breast cancer cell lysates. Under these conditions, ester-containing CD-nGels (NG2 and NG3) exhibited enhanced hydrolysis, while the fully amide-linked NG1 remained structurally intact. Rather than focusing on a disease-specific therapeutic model, a fluorescent dye Nile Blue (NB) was loaded via host-guest interactions as a fluorescent tracer. Upon cellular uptake by MCF-7 cells, we observed a direct correlation between chemical composition and intracellular behavior. The stable NG1 localized in compact patterns, while NG2 and NG3 displayed progressively diffuse distribution owing to reduced



Scheme 1 Schematic illustration of three CD-nGel formulations with progressively enhanced hydrolytic degradability. (Blue regions represent amide linkages, Orange regions represent ester linkages, circle-shaped regions indicate covalent CD-nGel linkages, and star-shaped regions denote crosslinking sites.).

stability. These results demonstrate that intracellular stability and distribution of CD-nGels can be tuned through rational chemical design, establishing a versatile platform for controlled-release drug delivery in future applications.

Materials and Methods

Materials

N-Isopropylmethacrylamide (NiPMAM, 97%), methacrylic anhydride (MA, $\geq 94\%$), acryloyl chloride (AC, $\geq 97\%$), BIS, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AMPA, $\geq 97\%$), N,N-dimethylformamide (DMF, $\geq 99.9\%$), triethylamine (TEA, $\geq 99.5\%$), C6 (98%), acetonitrile (MeCN, $\geq 99.9\%$) and deuterium oxide (D_2O , 99.9 atom%D), were purchased from Sigma-Aldrich. Nile Blue Acrylamide (NBAAm, $\geq 80\%$) and DEGDA were purchased from Polysciences. Beta-Cyclodextrin (βCD , 98%), ethanol (EtOH, 99%+), acetone (99.8%), and dimethyl sulfoxide (DMSO, 99.9%) were purchased from Thermo Fisher Scientific. Heptakis-(6-amino-6-deoxy)-beta-Cyclodextrin hepta-hydrochloride (HA- βCD , $\geq 98\%$) was purchased from CycloLab.

Modification of β -CD and HA- β CD with Vinyl Group

Synthesis of MA β CD

Methacrylation of HA- β CD was performed as previously described.^{67,68} MA (2.0 mL, 13.7 mmol) and 9 mL of DMF were added to a 50 mL round-bottom flask. HA- β CD (270.0 mg, 0.195 mmol) was dissolved in 3:2 (v/v) DMF/ H_2O mixture (15 mL). After deoxygenation under N_2 flow for 15 min, the HA- β CD solution was added dropwise to the MA solution under stirring (500 rpm). After stirring overnight, the reaction mixture was precipitated in an excess of cold acetone (800 mL), redispersed in Milli-Q water, and reprecipitated in acetone three times. The final product MA β CD was diluted in a small amount of water and vacuum freeze dried. The yield of MA β CD was 70%.

Synthesis of AC β CD

The incorporation of vinyl groups into β -CD was carried out according to previously reported methods.^{69,70} TEA was freshly distilled, and all glassware was flame-dried prior to use. β CD (1.5 g, 1.32 mmol) was dissolved in anhydrous DMF (11 mL) in a round-bottom flask, followed by the addition of TEA (400.7 mg, 3.96 mmol). The reaction mixture was stirred and degassed under a nitrogen (N_2) atmosphere and cooled to $0^\circ C$ in an ice bath. AC (477.9 mg, 5.28 mmol) was then added dropwise with continuous stirring (500 rpm). The reaction was allowed to proceed overnight, gradually

warming to room temperature. Upon completion, the precipitated white residue triethylamine hydrochloride salt (TEA·HCl) was removed by filtration. The clear filtrate was added into a large volume of acetone (450 mL) to precipitate the AC β CD. The precipitation was repeated three times with acetone. The final white solid was redissolved in water and lyophilized. The yield of AC β CD was 63%.

Preparation of Nile Blue (NB)-Labeled CD-nGels

NB-labeled CD-nGels with three different crosslinking approaches were synthesized via precipitation polymerization, following previously reported procedures.^{52,68,70} The synthesis of the fully amide-crosslinked nGel (NG1) is described here as a representative example: NiPMAm, MA β CD, and BIS were dissolved in 7.5 mL of Milli-Q water in a three-necked flask equipped with a reflux condenser. The mixture was degassed under N₂ for 15 min, after which the flask was placed in a preheated oil bath at 70°C. Subsequently, an aqueous solution of NBAAm (1.2 mM, 0.1 mol% of total monomers) was added to the reaction mixture. Polymerization was initiated by the dropwise addition of AMPA solution (6.5 mM, 0.7 mol% of total monomers). After 4 h of reaction, the mixture was cooled to room temperature and stirred overnight. The resulting product was purified via dialysis using a cellulose membrane (6–8 kDa molecular weight cutoff), first in EtOH for 6 h and then in Milli-Q water for 5 d with the water being changed 3 times daily. The purified nGel suspension was freeze-dried and stored at 4°C. For the synthesis of ester-bound CDs with amide-crosslinked nGel (NG2), and fully ester-crosslinked nGel with ester-based CDs (NG3), AC β CD, BIS, and DEGDA were used at the molar ratio shown in Table 1.

Each CD-nGel formulation was synthesized in three independent batches ($n = 3$) to evaluate reproducibility. After purification and lyophilization, the average reaction yields were $79.5 \pm 2.5\%$ (NG1), $76.5 \pm 2.9\%$ (NG2), and $80.7 \pm 3.7\%$ (NG3), reported as mean $\pm$ standard deviation (SD).

Characterization of Modified CD and CD-nGels

Nuclear Magnetic Resonance (NMR)

¹H NMR spectra were recorded with a Bruker 400 NMR spectrometer (Bruker, Rheinstetten, Germany) operating at 400 MHz. Briefly, 5 mg of purified MA β CD and AC β CD samples were dissolved in 700 μ L D₂O. The chemical shifts are given in parts per million (ppm).

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of the modified CDs and CD-nGels were acquired using an attenuated total reflection-FTIR (ATR-FTIR) spectrometer (IRTracer-100, Shimadzu, USA). Measurements were performed at a resolution of 2 cm⁻¹ over the spectral range of 4000–500 cm⁻¹.

Liquid Chromatography–Mass Spectrometry (LC–MS)

LC–MS analysis was conducted to determine the molecular weight of the modified CDs. Aqueous solutions of purified MA β CD and AC β CD (20 μ g/mL) were directly injected (30 μ L injection volume) in positive electrospray ionization (ESI⁺) mode using an Advion Expression LC-MS mass spectrometer (Advion, USA). The mobile phase consisted of acetonitrile/H₂O/formic acid (50:50:0.1, v/v/v), delivered at a flow rate of 0.3 mL/min.

Table 1 The Molar Ratio of the Components in the Nile Blue-Labeled CD-nGels

CD-nGel	Monomer			Crosslinker		Dye	Initiator
	NiPMAm	MA β CD	AC β CD	BIS	DEGDA	NBAAm	AMPA
NG1	99.5%	0.5%	0	5%	0	0.1%	0.7%
NG2	99%	0	1%	5%	0		
NG3	99%	0	1%	0	5%		

Fluorescence Emission Spectra Determination

The fluorescence emission spectra of NB-labeled CD-nGels and free NBAAm in H₂O were measured using a multimode microplate reader (BioTek Synergy H1, Agilent, USA). CD-nGels were suspended in H₂O at a concentration of 10 mg/mL, and free NBAAm was dissolved in H₂O at 50 μM. The excitation wavelength was set to 580 nm.

Dynamic Light Scattering (DLS) and Zeta Potential

The hydrodynamic diameter (D_h) and volume phase transition temperature (VPTT) of CD-nGels were measured by DLS using a Zetasizer ZS (Malvern Instruments) at a concentration of 1 mg/mL in Milli-Q water. VPTT measurements were performed in the temperature range of 20–70°C, with data recorded at 2°C intervals. Prior to each measurement, samples were equilibrated for 240 seconds to stabilize at the corresponding temperature. The volumetric swelling ratio (Q) of each NG was calculated based on the volume ratio of the NG in swollen (20°C) and collapsed state (70°C). Zeta potentials were detected at a concentration of 1 mg/mL in MilliQ-water. All DLS and zeta potential measurements were performed in triplicate.

Transmission Electron Microscopy (TEM)

The morphology of the CD-nGels was examined using a Philips CM120 transmission electron microscope equipped with a 4K CCD camera. Briefly, 5 μL of 0.3 mg/mL nGel suspension was deposited onto a carbon film-coated copper grid and negatively stained with 2% uranyl acetate prior to imaging.

Hydrolysis Study of CD-nGels

pH-Dependent Hydrolysis of CD-nGels

Three types of CD-nGels were dispersed in phosphate buffer (PBS, 10 mM, pH = 7.4) or acetate buffer (10 mM, pH = 5.1) and incubated at 37°C under gentle shaking to avoid sedimentation. At each time point, the mean count rate and D_h of each CD-nGels were measured with DLS with the fixed position and attenuator, to ensure consistent measurement conditions and minimize potential effects of refractive index variations. The pH of the suspension before and after incubation was recorded using a calibrated pH meter.

Degradation of CD-nGels by Cell Lysate

Michigan Cancer Foundation-7 (MCF-7) human breast cancer cell lysates were prepared in PBS to mimic intracellular hydrolytic conditions. Briefly, MCF-7 cell suspensions were placed in an ice bath and subjected to probe sonication (25 MHz, 45 s with 5 s on/5 s off pulse cycle) followed by centrifugation (4°C, 1,000 rcf, 10 min). The supernatant was collected as the active lysate. CD-nGel suspensions (1.5 mg/mL) were incubated with the cell lysate at 37°C in the presence or absence of phenol red. At selected time points, samples were analyzed using UV-vis spectroscopy. Phenol red was employed as a pH-sensitive indicator to monitor acidification of the medium resulting from ester bond cleavage, which is reflected by an increase in absorbance near 430 nm (A_1) accompanied by a corresponding decrease near 560 nm (A_2).⁷¹ To minimize interference from nGel scattering and lysate components, background corrections were performed as follows: (i) spectra of CD-nGels incubated in lysate without phenol red were recorded as a background control and subtracted from spectra of samples containing phenol red, and (ii) spectra of lysate with phenol red in the absence of CD-nGels were recorded and corrected using spectra of lysate alone to avoid the influence of cell lysate. To compare the shift between A_1 and A_2 , the integrated area of A_1 was normalized to A_2 , yielding a normalized area value denoted as A_1^* . The relative A_1^* area increase ratio over time was calculated according to the following equation:

$$\text{Area increased ratio}_{430\text{nm}} (\%) = \frac{(A_{1D20}^* - A_{1D0}^*)}{A_{1D0}^*} \times 100\%$$

where A_{1D20}^* and A_{1D0}^* represents the normalized 430 nm absorbance area after 20 days of incubation and at Day 0 (D_0), respectively. This parameter was used as a quantitative indicator of lysate-associated degradation of the CD-nGels.

CD-nGels Loading with Coumarin 6 (C6)

Each type of CD-nGel (10 mg) was suspended separately in 2 mL of Milli-Q water. C6, corresponding to 150 mol% relative to the theoretical amount of CD incorporated into the nGels, was dissolved in 200 μ L of DMSO. The CD-nGel suspension was added dropwise to the C6 solution under ultrasonication. Subsequently, the mixture was left to equilibrate on a shaker at 500 rpm at room temperature in the dark for 24 h. The resulting suspension was filtered to remove any precipitated C6 and dialyzed against Milli-Q water (6–8 kDa molecular weight cutoff of the membrane) overnight to reduce the DMSO concentration prior to freeze-drying. The products were freeze-dried to yield CD-nGels@C6 ICs (NG1@C6, NG2@C6, and NG3@C6). To determine the encapsulation capacity (EC) and absolute amount of C6 loaded per unit amount of CD-nGels (μ g/mg), 3 mg of each type of CD-nGel@C6 ICs was suspended in 1 mL of DMSO. A 200 μ L aliquot of this solution was further diluted with 3 mL of DMSO. The content of C6 in each sample was quantified using a UV–Vis spectrophotometer (Lambda 2, PerkinElmer). To exclude nonspecific absorption and scattering effects, a control experiment was conducted using p(NiPMAM)-BIS nanogels without CD. Meanwhile, empty nGels were used as controls to account for any background absorbance due to scattering. UV–Vis spectra of nGel@C6 samples were corrected by subtracting spectra of blank nGels at identical concentrations. Baseline correction was applied prior to quantification. The amount of loaded C6 was calculated according to a previously recorded calibration ([SI, Figure S1](#)). EC (%) and absolute amount of C6 loaded per unit amount of CD-nGels (μ g/mg) were calculated according to the following equations:

$$\text{EC}(\%) = \frac{\text{wt of C6 in CDnGels}}{\text{initial wt of C6}} \times 100\%$$

$$\text{Absolute amounts of C6}(\mu\text{g/mg}) = \frac{\text{wt of C6 in CDnGels}}{\text{wt of CDnGels@C6 complex}}$$

Cell Culture

MCF-7 cells were obtained from American Type Culture Collection (ATCC). MCF-7 was cultured in Minimum Essential Medium α (MEM α , Gibco), supplemented with 10% fetal bovine serum (FBS, Bodinco) and 1% penicillin/streptomycin (P/S, Gibco) in an incubator with 5% CO₂ at 37°C.

Cytotoxicity Assay

The cytotoxicity of CD-nGel on MCF7 was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The cells were seeded at a density of 5000 cells/well in a 96-well plate and cultured for 24 h. The culture medium was then replaced with fresh MEM α containing various concentrations (0.125–2 mg/mL) of CD-nGel incubated for another 24 h. Cells without treatment were used as control and MEM α medium as blank. Afterwards, the nGel suspension was aspirated and the cells were washed gently with PBS three times. Subsequently, 100 μ L of DMSO was added to each well. The crystals were dissolved by putting the plate on the shaker (10 min) until all the crystals were homogeneously dissolved. After gently shaking, the supernatant was transferred to another 96-well plate. The optical density (OD) of each well was measured at 570 nm using BioTek Synergy H1 microplate reader (Agilent, USA). Triplicates of each condition were used. The percentage of viable cells was calculated with the following equation:

$$\text{Cell viability}(\%) = \frac{\text{OD}_{\text{nGels}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}}} \times 100\%$$

Data were obtained from three independent biological replicates ($n = 3$), with each replicate representing experiments conducted on separate cell populations at different time points.

Cellular Uptake Experiment of CD-nGels@C6 ICs

The MCF-7 cells were seeded in 24-well plate at a density of 20000 cells/well and cultured for 24 h. Subsequently, the medium was removed, and the cells were treated with 0.5 mL of CD-nGel@C6 MEM α suspension at a concentration of 0.5 mg/mL under dark for 2 h and 24 h. Following this, the extra dispersion was removed, and the cells were washed with PBS three times. The colocalized images of the MCF-7 cells and CD-nGels@C6 were captured using a Zeiss

CellDiscoverer7 LSM900 microscope equipped with an environmental control chamber. The images were obtained from three independent experiments conducted at different time points.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism (version 10.4.0, GraphPad software). A minimum of three biological replicates were analyzed for quantitative analysis. All data were presented as the mean $\pm$ standard deviation (SD). The statistical significance of differences between two groups was determined using Student's *t*-test. Statistical significance was determined via two-way ANOVA for multiple comparisons.

Results

Synthesis and Characterization of CD-nGels

A series of CD-nGels with progressively enhanced hydrolytic degradability were synthesized via free radical precipitation polymerization. Prior to polymerization, two types of modified β CD, MA β CD (methacrylamide-functionalized) and AC β CD (acrylate-functionalized), were prepared from HA- β CD and native β CD, respectively. MA β CD was obtained by reacting the primary amine groups of HA- β CD with MA via a nucleophilic addition/elimination mechanism, forming stable amide linkages (Figure 1A). The chemical structure and the average degree of substitution (DS) of MA β CD were confirmed

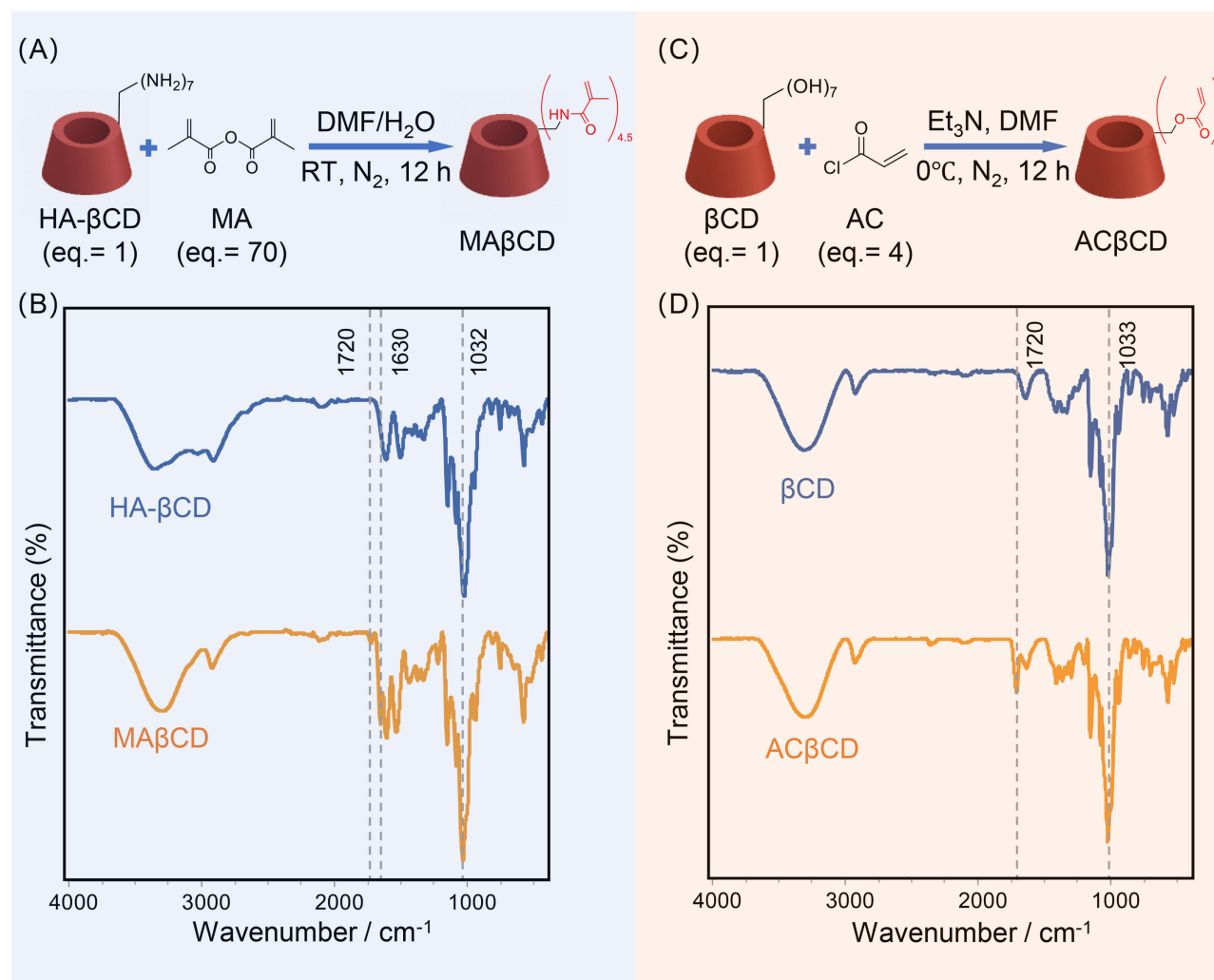


Figure 1 Synthesis and Characterization of the MA β CD and AC β CD. **(A)** Synthetic process of MA β CD, **(B)** FTIR spectra of HA- β CD and MA β CD **(C)** Synthetic process of AC β CD, **(D)** FTIR spectra of β CD and AC β CD.

by ^1H NMR, FTIR, and LC-MS analysis. In the FTIR spectra, both HA- β CD and MA β CD showed characteristic C–O–C vibration bands of the CDs' glucose units at 1032 cm^{-1} .⁷² Upon modification, a new band appeared at 1630 cm^{-1} , attributed to the C=O stretching vibration of the introduced amide groups, indicating successful conjugation.^{69,72} Additionally, a weaker signal at 1720 cm^{-1} was observed, corresponding to ester carbonyl stretching, suggesting that a small proportion of hydroxyl groups in HA- β CD also reacted with MA (Figure 1B).^{70,73} ^1H NMR and LC-MS further validated the molecular structure and degree of substitution. In the ^1H NMR spectrum of MA β CD, characteristic peaks at 5.6 ppm were assigned to the vinyl ($-\text{CH}=\text{CH}_2$) of the methacrylamide moieties (Figure 2).^{74,75} The DS of MA β CD was quantified by integrating the characteristic vinyl proton signals relative to the anomeric proton ($\text{C}_1\text{--H}$) of the β CD glucose units at 5.1 ppm.^{76–78} The anomeric proton signal serves as a reliable internal reference given its fixed stoichiometry of seven protons per β CD molecule and its well-resolved signal without significant peak overlap. Three independently synthesized batches were analyzed by ^1H NMR, yielding an average DS of 4.22 ± 0.11 (mean $\pm$ SD, $n = 3$). LC-MS analysis of MA β CD in positive electrospray ionization (ESI+) mode showed distinct molecular ion peaks at m/z 1399.8, 1467.8, and 1535.9, corresponding to HA- β CD conjugated with 4–6 methacrylamide groups (SI, Figure S3, calculated MWs: 1399.59, 1467.61, and 1535.64 g/mol), thereby confirming the expected modification pattern.

AC β CD was synthesized under alkaline conditions by reacting the hydroxyl groups of β CD with AC, yielding acrylate-functionalized CD (Figure 1C). FTIR spectra of AC β CD revealed the typical C–O–C stretching band of the glucose backbone at 1033 cm^{-1} , accompanied by a strong ester-carbonyl stretching vibration signal at 1720 cm^{-1} , indicating the successful incorporation of acrylate groups (Figure 1D).^{69,70,73} The ^1H NMR spectrum showed olefinic proton signals in the range of 6.0–6.5 ppm, further confirming the conjugation of acrylate groups (SI, Figure S4). The DS of AC β CD was similarly determined by ^1H NMR integral ratio method. Integration of three vinyl proton signals (6.5, 6.3, and 6.0 ppm) relative to the $\text{C}_1\text{--H}$ proton at 5.1 ppm resulted in an average DS of 1.92 ± 0.05 (mean $\pm$ SD, $n = 3$), based on three independently synthesized batches.

CD-nGels were synthesized via surfactant-free radical polymerization using the cationic initiator AMPA (Figure 2). Amide-functionalized MA β CD or ester-functionalized AC β CD was used as a co-monomer with NiPMAM to form the polymeric backbone. To construct the crosslinked 3D network, either amide-based BIS or ester-based DEGDA was employed as the crosslinker. A small amount of the fluorescent dye NBAAM was also incorporated covalently to label the CD-nGels, enabling further tracking during cell experiments. By varying the combinations of CD-based monomers and crosslinkers, three types of fluorescent CD-nGels with progressively increased hydrolytic degradability were obtained. NG1 (MA β CD–BIS) contained exclusively amide linkages at both the CD and crosslinking sites. NG2 (AC β CD–BIS) featured ester linkages on the CD component and amide linkages in the crosslinker. NG3 (AC β CD–DEGDA), expected to exhibit the highest hydrolytic sensitivity, incorporated ester linkages at both the CD and crosslinking sites.

After polymerization, the NB-labeled fluorescent CD-nGels were dialyzed against ethanol and water to remove unreacted monomers and impurities, followed by freeze-drying and storage at 4°C . To evaluate the incorporation of amide- and ester-functionalized CDs into the nGel network, FTIR spectroscopy was performed. As shown in Figure 3A, all three types of CD-nGels exhibited a characteristic C–O–C stretching vibration at $\sim 1035\text{ cm}^{-1}$, corresponding to the glucose units of β CD, confirming the successful CD incorporation. In the carbonyl region ($1600\text{--}1800\text{ cm}^{-1}$), distinct spectral differences were observed depending on the type of chemical linkage in the nGels composition. NG1 showed a single absorption band at $\sim 1630\text{ cm}^{-1}$, attributed to the amide C=O stretching from both the NiPMAM backbone and the MA β CD/BIS components. In contrast, NG2 exhibited a dominant amide-carbonyl band at 1630 cm^{-1} along with a weaker band at $\sim 1720\text{ cm}^{-1}$, indicating the presence of ester linkages introduced by AC β CD. NG3, containing both ester-functionalized AC β CD and ester-based crosslinker DEGDA, showed a more prominent ester C=O stretching band at $\sim 1720\text{ cm}^{-1}$. These results are consistent with the designed nGel compositions: NG1 contains only amide-based linkages, NG2 contains ester-functionalized CDs and amide crosslinkers, and NG3 contains ester linkages in both CD moieties and crosslinkers.

The zeta potentials of NG1, NG2, and NG3 (three independent batches, $n = 3$) were determined to be $+20.2 \pm 1.2\text{ mV}$, $+15.4 \pm 1.3\text{ mV}$, and $+12.0 \pm 1.0\text{ mV}$, respectively (Figure 3B). Due to the use of the cationic initiator AMPA and the

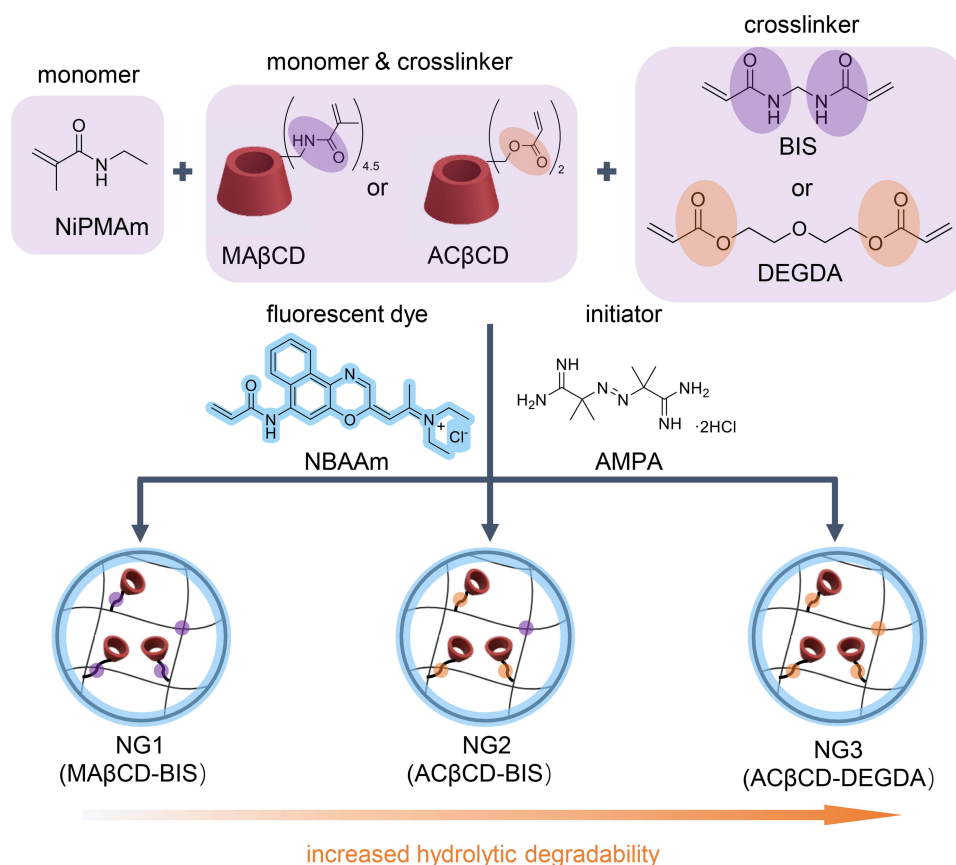


Figure 2 Schematic illustration of three types of CD-nGels with progressively enhanced hydrolytic degradability. Two types of modified β CD, amide-linked MA β CD and ester-linked AC β CD, were employed as co-monomers. Additionally, amide-based BIS and ester-based DEGDA served as crosslinkers. NBAAm was incorporated to introduce fluorescent labeling into the CD-nGels. (Purple circle-shaped regions indicate amide linkages, while Orange circle-shaped regions represent ester linkages.).

incorporation of NBAAm, all CD-nGels exhibited a positive surface charge. Among the three formulations, NG1 showed the highest zeta potential, which is attributed to the presence of residual amino groups on the glucose units of MA β CD.

Particle size and morphology were characterized by DLS and TEM, which provide complementary information on hydrodynamic diameter in dispersion and morphology in the dry state. This combination is widely employed for nGel characterization in soft colloidal systems.^{79–82} The hydrodynamic diameters (D_h) of CD-nGels synthesized in three independent batches were evaluated by DLS (Figure 3C). The mean D_h values determined were 247 ± 11 nm (NG1), 431 ± 16 nm (NG2) and 363 ± 10 nm (NG3), (mean $\pm$ SD, $n = 3$), demonstrating good batch-to-batch consistency and indicating the influence of monomer and crosslinker composition on particle size. Additionally, all three CD-nGels exhibited PDI values below 0.2, indicating a monodisperse system.^{83–85} The morphology of the synthesized CD-nGels was examined by TEM. As shown in Figure 3D, all three types of CD-nGels revealed homogeneous spherical structures with well-defined boundaries.

The successful incorporation of the fluorescent dye NBAAm into the CD-nGels was further confirmed by fluorescence emission spectroscopy (SI, Figure S5). Free NBAAm in aqueous solution exhibited a maximum emission signal at 685 nm under 580 nm excitation. In contrast, emission peaks of CD-nGels shifted towards shorter wavelength (blue-shifts) ranging from 660–670 nm. This blue-shift can be attributed to the less polar environment of the covalently bound NB dye within the polymeric network compared to the free NB in water, confirming the successful labeling of the nGels with NBAAm.⁸⁶

The temperature-dependent D_h of the CD-nGels was determined by DLS over a temperature range of 20–70°C. Due to the thermoresponsive nature of the poly(NiPMAM) backbone, which has a LCST of approximately 44°C, all three CD-nGels exhibited a similar volume phase transition temperature (VPTT) of 42–44°C (SI, Figure S6A). Above this

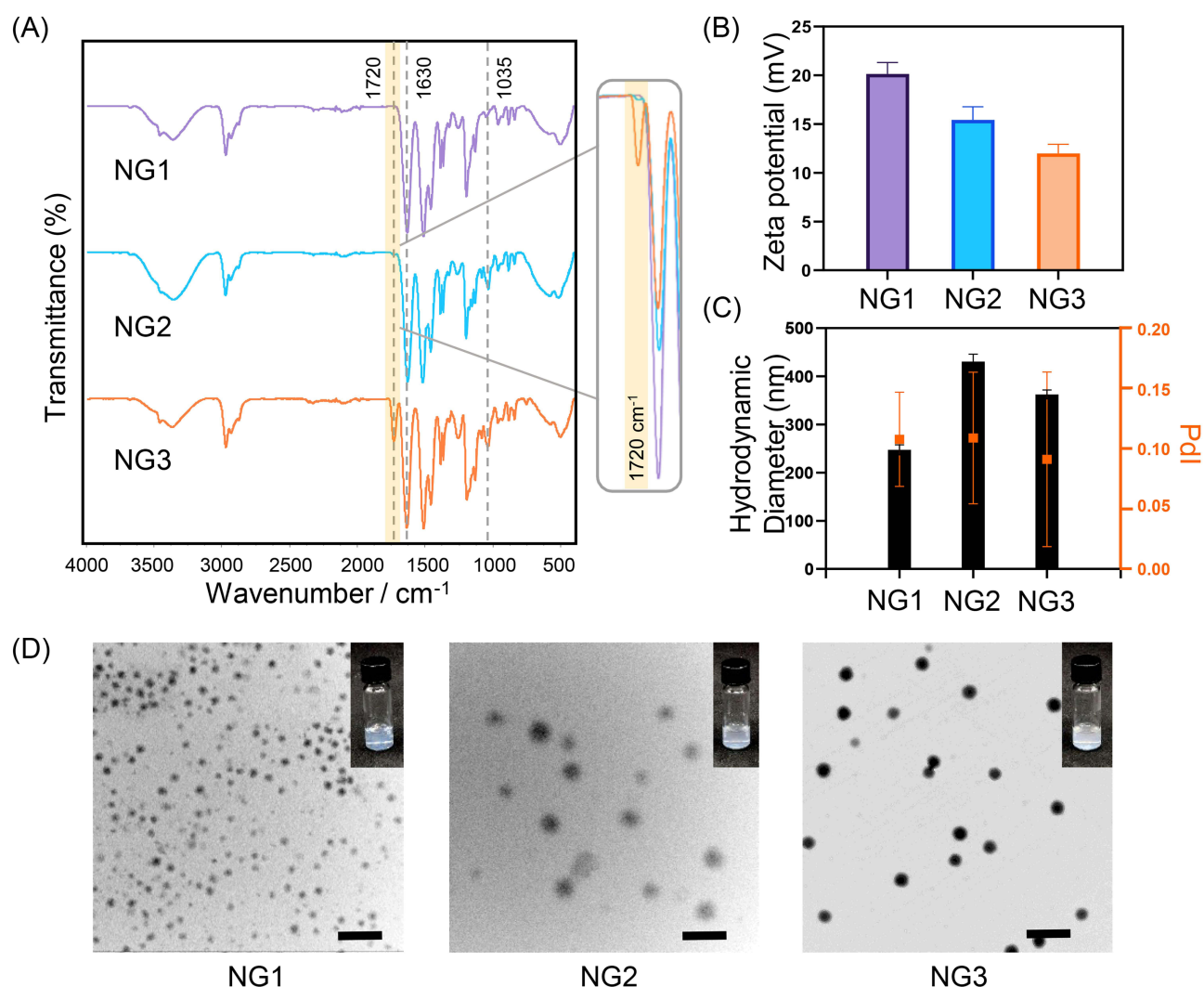


Figure 3 Characterization of the CD-nGels. **(A)** FTIR spectra of three types CD-nGels (NG1, NG2, and NG3), with the 1600–1800 cm^{-1} region zoomed in for clarity. **(B)** Zeta potentials of CD-nGels in Milli-Q water (concentration of CD-nGels: 1 mg/mL) **(C)** D_h and PDI of CD-nGels. **(D)** TEM images of CD-nGels; insets show photographs of CD-nGel aqueous suspensions. Scale bar = 1 μm .

transition temperature, the polymeric network undergoes a coil-to-globule transition, resulting in the collapse and shrinkage of the nGels in aqueous solution.⁸⁷ To assess the crosslinking density of each CD-nGel, the volumetric swelling ratio (Q) was calculated by comparing the volumes in swollen (20°C) and collapsed state (70°C) (Figure 4).^{88,89} Since crosslinking density indicates the flexibility of the polymeric network, it is directly reflected in the swelling behavior of nGels.^{89–91} NG1 exhibited the highest swelling ratio ($Q = 9.0$), while NG2 and NG3 showed relatively lower values ($Q = 5.6$ and 4.7 , respectively). These lower swelling ratios suggest a more rigid network and a higher effective crosslinking density in NG2 and NG3. Although the feed ratio of the primary crosslinkers (BIS or DEGDMA) was kept constant for all formulations, the variations in CD content also influenced the stiffness. NG1 contained 0.5 mol% MA β CD, while NG2 and NG3 incorporated 1 mol% AC β CD. Since these CD derivatives possess multiple reactive vinyl groups, they act as additional crosslinking sites, thus increasing the overall crosslinking density of the resulting CD-nGels.

Hydrolysis of CD-nGels Governed by Cross-Linking Nature and Hydrolytic Conditions

Three CD-nGels with distinct chemical linkages were applied in this study. NG1 consisted of non-degradable amide-linked β CD (MA β CD) crosslinked with BIS, whereas NG2 and NG3 contained ester-linked β CD (AC β CD) combined with BIS or ester-based crosslinker DEGDA, respectively. Their hydrolytic stability was monitored over 30 days under physiological (pH = 7.4) and mildly acidic conditions (pH = 5.1) by recording the time-dependent scattering intensity (count rate) using DLS. Because scattering intensity is proportional to particle concentration, the loss of structural integrity due to nGel hydrolysis is expected to reduce the measured count rate.^{79,92} As shown in Figure 5A and B, at pH 7.4, all three CD-nGels maintained their structural integrity, showing negligible variation in scattering intensity over time. Specifically, the relative count rate of NG1 (MA β CD-BIS) remained virtually unchanged (101.0%), NG2 (AC β CD-BIS) showed a slight reduction to 98.9%, and NG3 (AC β CD-DEGDA) decreased only to 93.1% after 30 days, indicating high colloidal stability in neutral conditions. In contrast, under acidic conditions (pH 5.1), distinct degradation behaviors were observed among various formulations. NG1 exhibited excellent hydrolytic resistance, retaining 96.0% of its initial count rate after 30 days, consistent with its fully amide-based network. NG2 displayed moderate degradation with a relative count rate decrease to 88.3%, while NG3 underwent the most pronounced degradation, with its count rate reduced to 76.5% over the same period. This accelerated degradation of NG3 can be attributed to the presence of acid-labile ester linkages in both the AC β CD units and the DEGDA crosslinker. Hydrolysis of these bonds disrupted the crosslinked network, generating carboxylic acid groups that not only contributed to network dissociation but also further catalyzed ester cleavage in a more acidic environment.^{93,94} The resulting decrease in particle concentration led to a reduced scattering intensity in DLS measurements.^{79,92} To exclude the possibility that change in scattering intensity

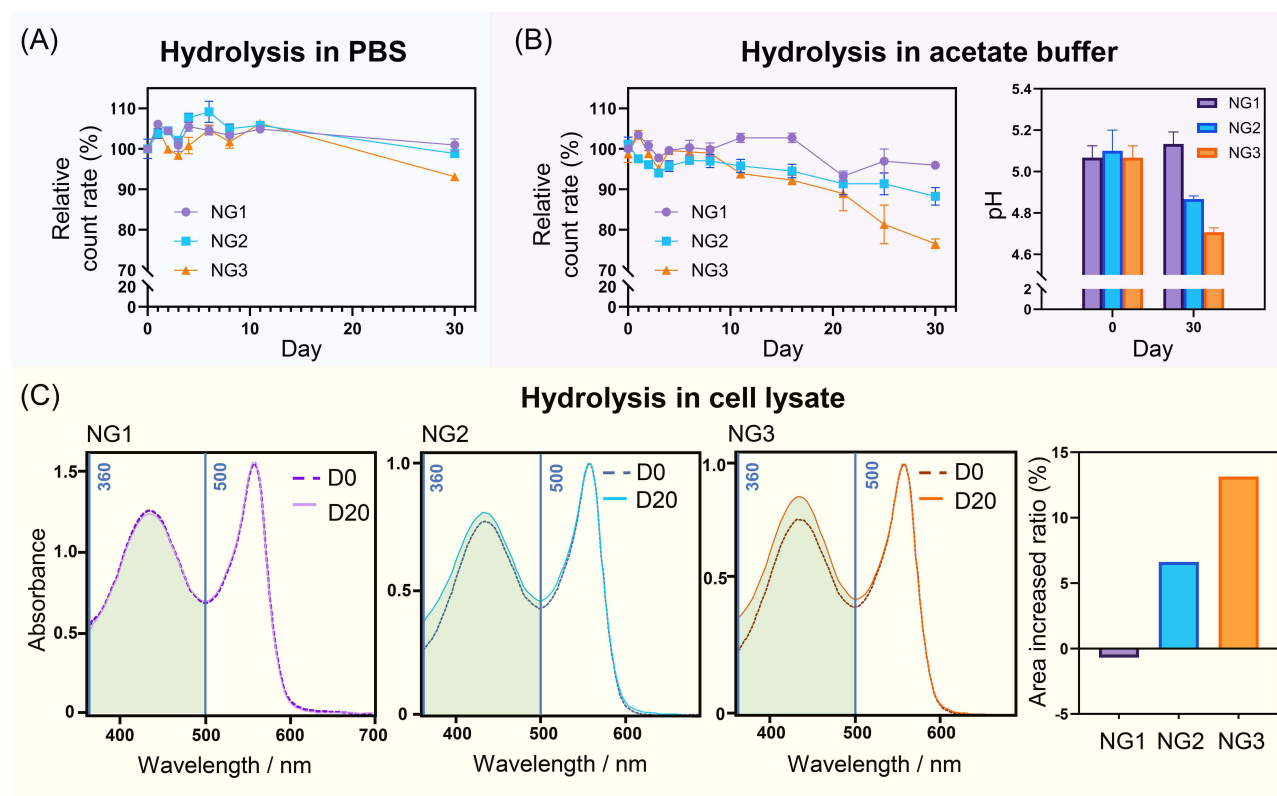


Figure 4 Cell viability of NG1, NG2, and NG3. MCF-7 cells were treated with CD-nGels for 24 h at 37°C and the cytotoxicity was determined by MTT assay. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Tukey post hoc test. P values: * $p < 0.05$.

originated from particle aggregation or sedimentation, the size change was monitored in parallel (SI, Figure S7). The average D_h of all three CD-nGels remained statistically unchanged over time under both physiological and acidic conditions. This stability confirms the absence of aggregation, suggesting that the observed decrease in count rate is more likely associated with reduced network integrity. Such behavior is characteristic of a bulk degradation mechanism.^{95–97} Due to the hydrophilic nature of the pNiPMAM backbone and the integrated CD units, the polymer matrix is rapidly hydrated upon exposure to aqueous media, allowing water to penetrate throughout the network. During bulk degradation, internal bond cleavage reduces network connectivity while the global particle structure remains intact. As a result, D_h can remain relatively constant or even slightly increase as the loosening network imbibes additional water.⁹⁶ The pH of the CD-nGels suspension was also monitored before and after the hydrolysis experiments. In neutral PBS, no significant pH changes were observed for any of the nGel formulations (SI, Figure S8). However, under acidic conditions, the final pH decreased slightly to 4.9 for NG2 and 4.7 for NG3 as a consequence of productions of carboxylic acid groups during ester hydrolysis. This trend correlated well with the DLS results, further confirming the acid-catalyzed cleavage of ester bonds and degradation of ester-containing CD-nGels.

To evaluate network stability under intracellular-mimicking conditions, CD-nGels were incubated in MCF-7 cell lysate with phenol red as a pH indicator. Due to the pH-responsive nature of phenol red, acidification of the medium, arising from ester-bond cleavage and generations of carboxylic acid groups, resulted in a decrease in absorbance at 560 nm (A2) and an increase at 430 nm (A1).^{98,99} To exclude potential interference from background scattering or lysate components, a series of control groups was set-up. Spectra of CD-nGels incubated in phenol red-free lysate were recorded and subtracted from the experimental spectra to correct background scattering. Additionally, the stability of lysate was monitored over the same incubation period with phenol red indicator in the absence of CD-nGels. To quantify the spectra shifts while reducing baseline drift and concentration-dependent effects, the integrated area of the 430 nm band was normalized to that of the 560 nm band, yielding a normalized area value ($A1^*$). The corrected and normalized spectra recorded at Day 0 and after 20 days of incubation, together with the relative area increased ratio of $A1^*$ (%), are shown in Figure 5C. Specifically, NG1, which is entirely amide-bound, showed negligible changes in $A1^*$ (−0.7%) over 20 days, indicating high stability under intracellular-mimicking conditions. NG2, containing ester-functionalized AC β CD but an amide-based BIS crosslinker, showed a moderate increase of 6.6% in $A1^*$ demonstrating partial lysate-assisted cleavage of the ester bonds. In contrast, NG3, incorporating ester linkages both in the β CD units and in crosslinkers, exhibited a pronounced increase of 13.1% in $A1^*$ consistent with its more ester-rich structures. In this lysate control experiment (SI, Figure S9), the normalized $A1^*$ value increased by only around 1.4% after 20 days, indicating that the lysate itself does not induce significant spectral changes under the experimental conditions.

While DLS count-rate variations and phenol red spectral shifts serve as indirect indicators of nGel hydrolysis rather than direct measures of bond cleavage, the data are internally consistent and remain highly compelling. The clear correlation between the various chemical composition of the CD-nGels (amide versus ester linkages), the loss of network integrity, and the acidification of the surrounding medium confirms that the ester-containing CD-nGels are more susceptible to hydrolysis under acidic and intracellular-mimicking conditions.

Loading C6 into CD-nGels

To enable real-time tracking of intracellular drug delivery and release, C6, a hydrophobic model drug with intrinsic fluorescence, was loaded into CD-nGels to form CD-nGels@C6 ICs (binding constant $K \approx 218.88 \text{ M}^{-1}$).¹⁰⁰ In this system, β CD units embedded within the nGel network serve as host molecules, while hydrophobic C6 molecules act as the guest, occupying the hydrophobic cavity of β CD. The β CD@C6 ICs form in a 1:1 stoichiometry via noncovalent interactions and specific structural relationship such as molecular recognition.^{101–103} To enhance the absolute amount of C6 loaded into the nGel system, 150 mol% C6 relative to the theoretical number of CD moieties incorporated into the CD-nGels was added during the loading procedure. As shown in Figure 6A, after 24 h incubation, the resulting CD-nGels@C6 ICs was filtered to remove precipitated free drugs and dialyzed against Milli-Q water overnight. The purified products were then freeze-dried to yield three types of C6-loaded nGels (NG1@C6, NG2@C6, and NG3@C6). The EC % and absolute amounts of C6 loaded per unit amount of CD-nGels ($\mu\text{g}/\text{mg}$) were quantified using UV-Vis absorption spectroscopy based on a pre-established C6 calibration curve (SI, Figure S1). The UV-Vis spectra of CD-nGels@C6 ICs

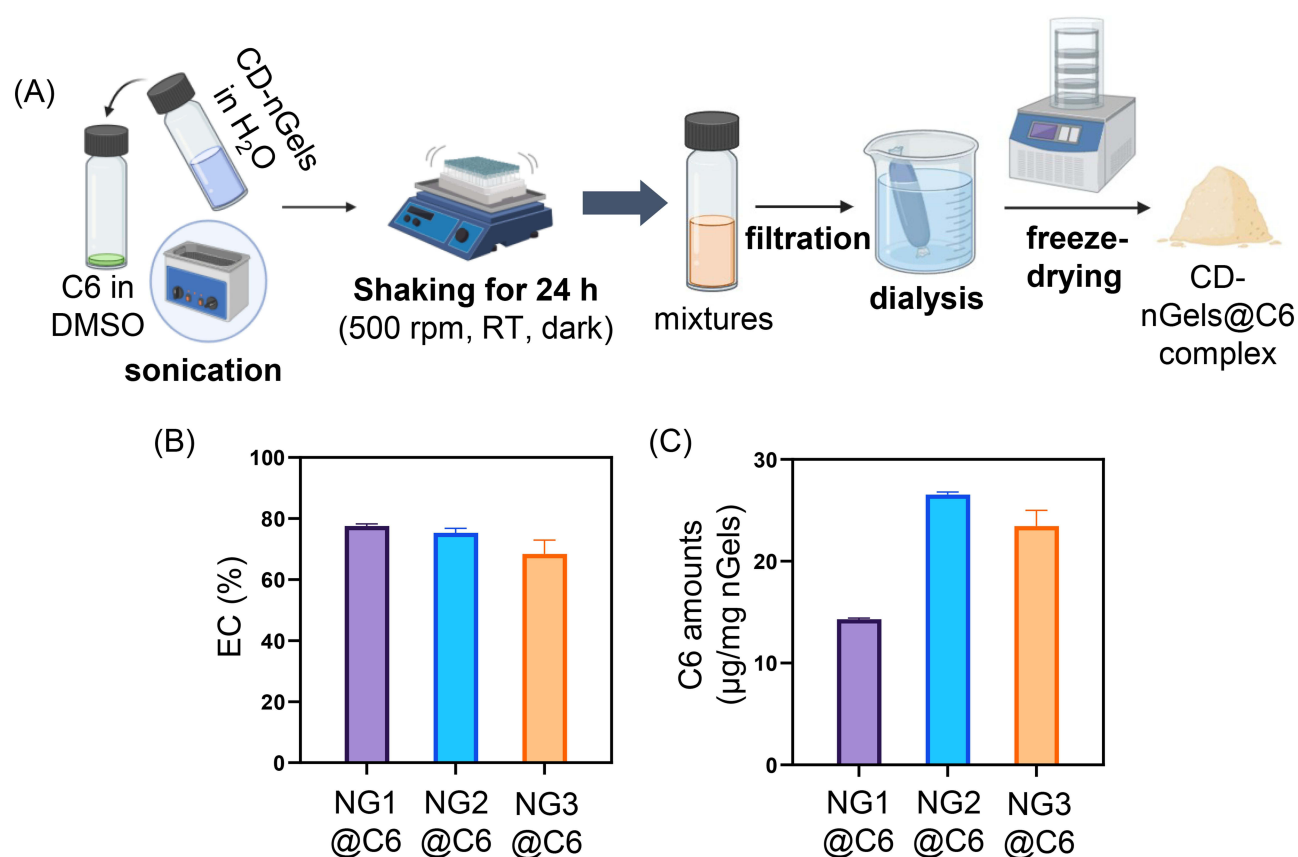


Figure 5 Hydrolysis study of CD-nGels. **(A)** Relative count rate (%) of CD-nGels in PBS (pH 7.4) over time. **(B)** Relative count rate (%) of CD-nGels in acetate buffer (pH 5.1, left), and corresponding pH changes before and after hydrolysis (right). **(C)** Degradation study of NG1, NG2, and NG3 in MCF-7 cell lysate, monitored by UV-vis spectroscopy. Normalized spectra are shown for each sample. Dashed lines represent the initial spectra at Day 0 (D0), while solid lines correspond to spectra collected at Day 20 (D20). The green-shaded region highlights the absorbance peak area centered at 430 nm (integrated from 360–500 nm). The relative increase in the 430 nm peak area (normalized to the 560 nm reference band) is summarized as bar graph, reflecting the extent of nGel hydrolysis under intracellular-mimicking conditions.

in DMSO exhibited a distinct absorption peak at 468 nm, which closely matched the characteristic absorption of free C6, confirming the successful incorporation of C6 into the CD-nGels (SI, Figure S10). The average calculated EC% values for NG1@C6, NG2@C6, and NG3@C6 were 77.6%, 75.3%, and 68.4%, respectively (Figure 6B). Correspondingly, the average absolute loading amounts were 14.3, 26.6, and 23.4 μg of C6 per mg of CD-nGels, respectively (Figure 6C). To assess the contribution of non-specific adsorption, a control experiment was conducted using CD-free p(NiPMAm)-BIS nGels. The average loading amount of C6 in this system was 7.7 $\mu\text{g}/\text{mg}$, which is significantly lower than that of CD-nGels. This result indicates that C6 loading is primarily driven by host-guest interactions with the incorporated CD moieties rather than nonspecific adsorption onto the polymer network. Notably, while the EC% values remained relatively consistent across the three CD-nGels systems (68% to 78%), NG2 and NG3 exhibited higher absolute loaded C6 amounts per unit mass, indicating a better loading performance for hydrophobic molecules. This difference can primarily be attributed to the higher β -CD content in the CD-nGels formulations: NG1 contained only 0.5 mol% β -CD relative to the total monomer content, whereas NG2 and NG3 incorporated 1 mol% β -CD. The increased availability of CD cavities in NG2 and NG3 provided more binding sites for the hydrophobic C6 molecules, resulting in enhanced drug loading.^{104,105}

Cytotoxicity Study of CD-nGels

To establish the biosafety profile of the nanocarriers prior to investigating their intracellular behavior, the cytotoxicity of blank CD-nGels was evaluated in MCF-7 cells via MTT assay (Figure 6). Given the established biocompatibility of C6,^{106,107} the testing of C6 loaded nGel was deemed unnecessary. A 24-h exposure period was selected for cytotoxicity

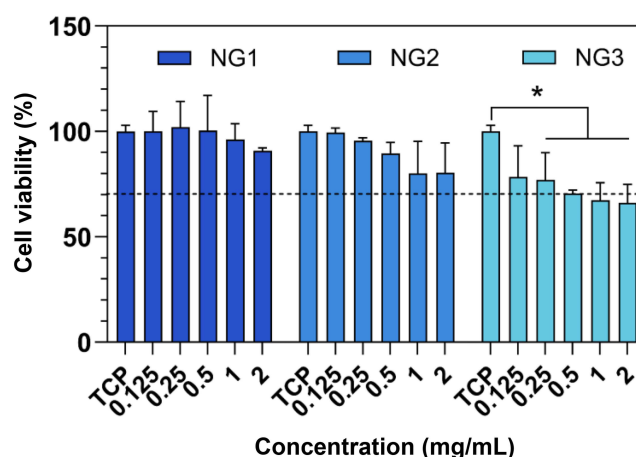


Figure 6 Preparation and characterization of CD-nGels@C6 ICs. (A) Schematic illustration of the preparation and purification process of CD-nGels@C6 ICs. (B) EC (%) of C6 in the three types of CD-nGels. (C) Absolute amount of C6 loaded per unit amount of CD-nGels ($\mu\text{g}/\text{mg}$), calculated based on the calibration curve.

assessment, adhering to ISO 10993–5 guidelines for initial in vitro biocompatibility screening. This duration also aligned with the co-incubation timeframes used for the subsequent cellular uptake investigations. Cytotoxicity assays revealed that NG1 crosslinked with all-amide bonds exhibited the lowest toxicity and the highest cell viability. NG2, crosslinked with a mixture of amide and ester bonds, demonstrated a slight decrease but non-significant decrease in survival. In contrast, the all-ester crosslinked NG3 exhibited a significant concentration-dependent reduction in cell viability, particularly at 1 and 2 mg/mL. This trend likely correlates with the hydrolysis of ester bonds in NG2 and NG3 networks, leading to acidification of the culture medium,^{56,61} as supported by the pH drop observed during our in vitro degradation studies. Conversely, the amide-linked NG1 remained stable and did not induce measurable pH changes. Notably, except for NG3 at 1 and 2 mg/mL, all CD-nGels maintained cell viability above 70% across the tested concentration range, thereby satisfying the cytotoxicity requirements of ISO 10993–5:2009. Consequently, while NG1 and NG2 are considered non-cytotoxic up to 2 mg/mL, NG3 is deemed safe for use at concentrations up to 0.5 mg/mL.

This outcome suggested that the chemical nature of the crosslinked structure exerts a substantial influence on the cellular toxicity of nGels. The well-established biosafety provides a favorable foundation for subsequent endocytosis studies. Future studies will conduct multifaceted cytotoxicity analyses by incorporating long-term cell viability measurements, apoptosis pathway analysis, and membrane integrity assessment, enabling a more comprehensive understanding of the material's safety profile and biological responses under repeated or prolonged exposure.

Cellular Uptake Study of CD-nGels@C6 ICs

Confocal fluorescence imaging analysis of CD-nGels@C6 ICs in MCF-7 cells revealed that intracellular behavior of these nanocarriers is mainly decided by their chemical structure. Specifically, the choice between amide and ester linkages significantly influenced both the structural integrity and the distribution patterns of the CD-nGels after cellular uptake. No visible aggregation or precipitation of nGels was observed in the culture medium during the incubation of nGels@C6 ICs with MCF-7 cells. This observation is also consistent with the thermoresponsive behavior of the p(NiPMAM) backbone of CD-nGel networks, whose VPTT lies above 37°C; therefore, temperature-induced collapse or dehydration is not expected under the conditions used for cellular uptake experiments.⁵² Following a 2-h incubation (Figure 7), all three CD-nGels (NG1@C6, NG2@C6, and NG3@C6) were efficiently taken up by the MCF-7 cells. The NB-labeled CD-nGels (red fluorescence) demonstrated discrete fluorescence signals, reflecting a non-homogeneous intracellular distribution within localization within vesicular compartments, such as endosomes or lysosomes. Notably, the fully-amide NG1 remains structurally intact upon internalization, corresponding well with our previous study.⁵² In this group, the loaded C6 (green fluorescence) remained localized near the nucleus with moderate intensity, suggesting limited cargo release during the early stages of uptake, which is consistent with higher structure stability of NG1. In contrast, NG3@C6, which contains ester linkages in both the CD moieties and crosslinking domains, displayed a more

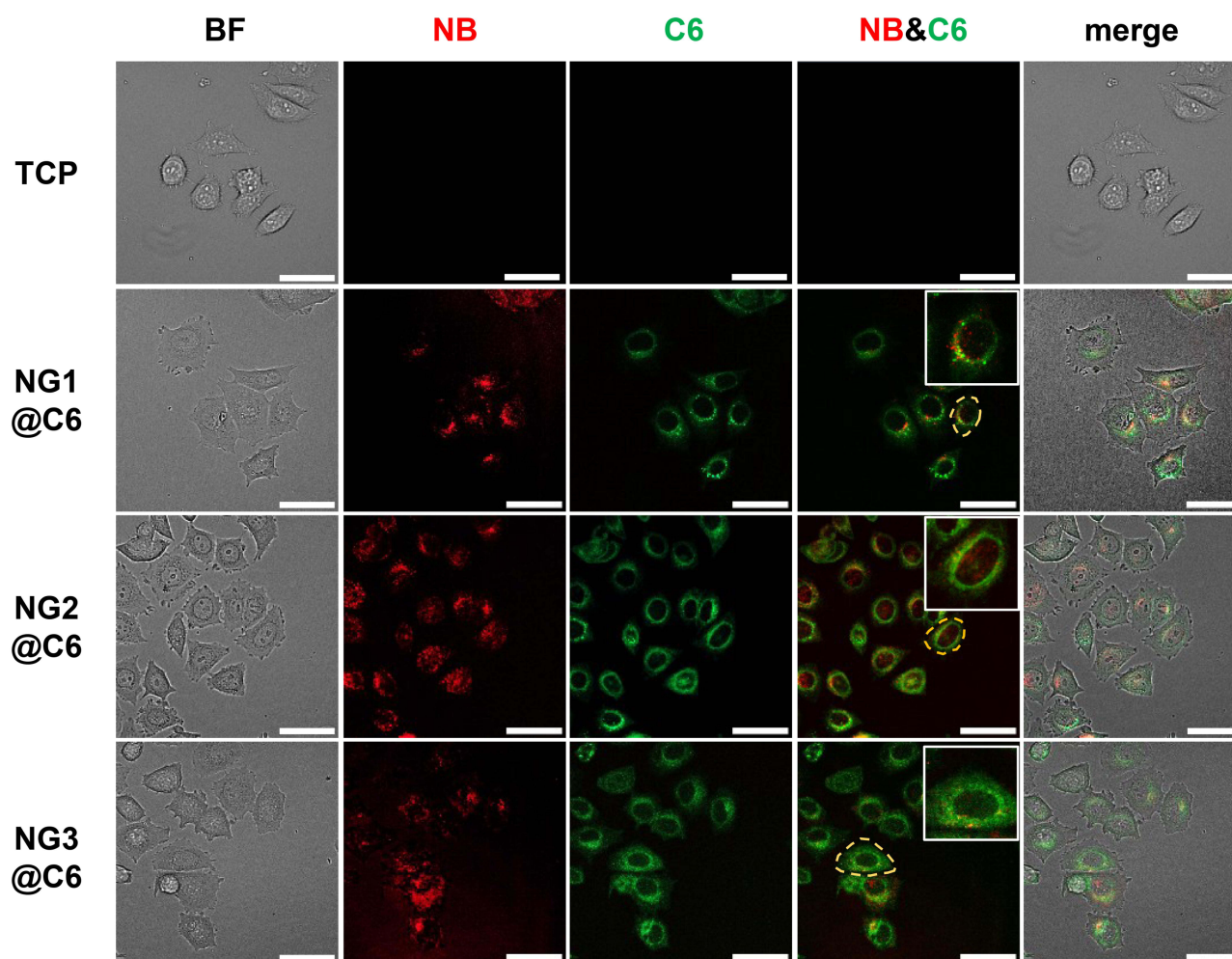


Figure 7 Fluorescence images of the colocalization of MCF-7 cells, NB (nGel), and C6. The MCF-7 cells were incubated with the NG1@C6, NG2@C6, and NG3@C6 for 2 h at 37°C and images were obtained using Zeiss CellDiscoverer7 LSM900 microscope. Images were enhanced for brightness and contrast uniformly across the entire field. The insets provide enlarged visualization of NB@C6 colocalization from cells with yellow circles. The enlarged areas are indicated by white squares. Scale bar = 50 μ m.

diffuse intracellular fluorescence pattern. This suggests that the ester-rich networks are more susceptible to intracellular environments, undergoing a more rapid degradation and became less compact upon uptake,⁵¹ which facilitates a broader intracellular distribution of the CD-nGels.

Upon extending the incubation period to 24 h, the differences in intracellular stability among the three CD-nGel formulations became even more pronounced (Figure 8). The red fluorescence of NG1 remained in a distinct punctate pattern localized in the perinuclear region, confirming the sustained structural integrity of all-amide network. In comparison, NG2 showed a transition toward a partially diffuse pattern interspersed with residual punctate spots, suggesting intermediate network stability. NG3@C6 exhibited a markedly diminished and highly diffuse signal, with both the nGel-associated red fluorescence and the loaded C6 distributed throughout the cytoplasm. These qualitative observations suggest that ester-rich CD-nGels undergo more extensive intracellular disassembly compared to the more stable fully amide-linked system, thus losing their structure intact and distributed throughout the cell over time.

In summary, the inherent differences in chemical stability between amide and ester linkages allow these CD-nGels to exhibit tunable intracellular behavior and distinct spatial distributions following endocytosis. The amide-linked NG1 maintained high structural integrity and presented a more compact intracellular distribution within the perinuclear region. Conversely, the ester-containing NG2 and NG3 displayed progressively diffuse cytoplasmic patterns, indicating the chemistry-dependent network degradations. These findings highlight how the rational chemical design of nGel

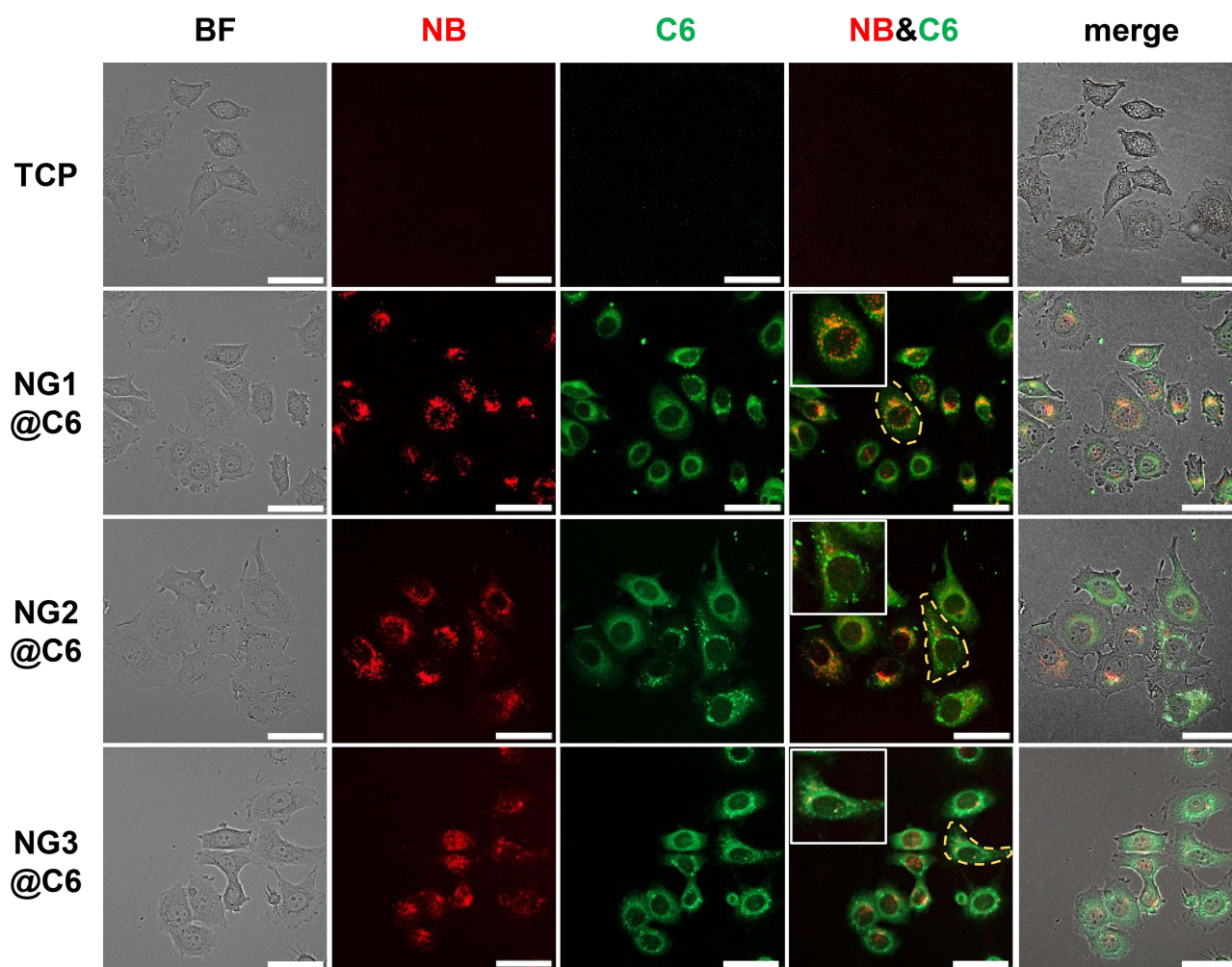


Figure 8 Fluorescence images of the colocalization of MCF-7 cells, NB (nGel), and C6. The MCF-7 cells were incubated with the NG1@C6, NG2@C6, and NG3@C6 for 24 h at 37°C and images were obtained using Zeiss CellDiscoverer7 LSM900 microscope. Images were enhanced for brightness and contrast uniformly across the entire field. The insets provide an enlarged visualization of NB@C6 colocalization from cells with yellow circles. The enlarged areas are indicated by white squares. Scale bar = 50 μ m.

architectures can modulate their intracellular behavior, establishing a solid foundation for future studies on controlled intracellular drug delivery.

Discussion

The design and functionalization of CD-based monomers played a critical role in enabling both polymer network formation and the tunable degradability of CD-nGels. In this work, amide-functionalized MA β CD and ester-functionalized AC β CD with multiple reactive vinyl groups were successfully synthesized. Subsequently, CD-nGels were synthesized via surfactant-free, free radical precipitation polymerization conducted entirely in aqueous conditions.^{68,70} In this approach, the growing thermoresponsive polymer chains undergo a coil-to-globule transition above their LCST, leading to the chain collapse and formation of stable precursor particles^{16,87} To facilitate this, the thermos-sensitive co-monomer NiPMAM was polymerized at 70°C along with the CD monomers. This temperature ensures the efficient thermal decomposition of the water-soluble azo-initiator AMPA while remaining well above the LCST of p(NiPMAM) (~44°C). Due to the numerous vinyl groups, both MA β CD and AC β CD functioned not only as monomers but also crosslinkers along with BIS/DEGDA. Given that functional co-monomers can significantly influence particle nucleation and internal network architectures,¹⁰⁸ their molar fractions were kept at 0.5–1 mol% to ensure reproducibility while preserving sufficient host–guest functionality. For the amide-based system, BIS was selected at

a 5 mol% feeding ratio to balance structural integrity with network flexibility, consistent with established protocols.^{68,109,110} Regarding the ester-based crosslinkers, DEGDA was selected due to its superior aqueous solubility (10–50 mg/mL). Unlike other commonly used ester-based crosslinkers such as ethylene glycol dimethacrylate (EGDMA), which require organic co-solvents (eg, 1,4-dioxane) due to poor aqueous solubility (<1 mg/mL), DEGDA allows for a purely aqueous synthesis, thereby avoiding the risk of residual organic solvent toxicity. All three synthesized CD-nGels exhibited uniform size distributions and good batch-to-batch consistency. The intrinsic nature of the chemical linkage, amide and ester, offered a rational approach to tuning the hydrolytic stability of the resulting nGels. Amide bonds in MA β CD confer higher stability, whereas the ester bonds in AC β CD are more susceptible to cleavage under acidic or enzymatically active environments, such as those found in the tumor microenvironment.^{31,52,111}

Hydrolytic stability is a critical determinant of nGel performance in biological applications. In this work, we systematically compared the hydrolytic behavior of three CD-nGels with distinct chemical structures. Observations from hydrolysis study in PBS (pH 7.4) and mildly acidic buffer (pH 5.1) highlight the higher susceptibility of ester linkages to acid-catalyzed hydrolysis, enabling controlled degradation in mildly acidic environments, such as those encountered in endosomal and lysosomal compartments, while remaining stable under physiological pH conditions.

MCF-7 cells were selected as a representative epithelial breast cancer model for proof-of-concept evaluation of nGel stability and intracellular behavior. This cell line is widely utilized for *in vitro* studies,^{112–114} and its use here ensures consistency with the previous intracellular tracking study in our group,⁵² providing a reproducible environment for both lysate-based degradation assays and fluorescence microscopy. To evaluate the nGel stability in the intracellular-mimicking environments, we monitored pH changes in MCF-7 cell lysates during co-cultures using phenol red as a spectroscopic indicator. Compared to traditional electrode-based pH-metry, this optical approach offers rapid equilibration and highly sensitive detection of degradation-induced acidification, even at low nGel concentrations.^{115,116} The degradation profiles observed in intracellular-mimicking MCF-7 cell lysates closely align with the trends found under acidic buffer conditions, confirming that our CD-nGel stability is inherently chemistry-dependent. While this methodology does not directly identify specific cleavage products, the strong correlation between ester content and the accelerated degradation behavior supports the conclusion that ester-rich networks exhibit reduced stability compared with their all-amide counterparts.

The intracellular study of three CD-nGels@C6 ICs further highlights the profound impact of network chemistry on both intracellular stability and distribution. The ester-containing CD-nGels (NG2 and NG3) displayed reduced structural stability compared to the all-amide system, consistent with the higher susceptibility of ester bonds to the acidic and enzyme-rich intracellular environment. As a result, NG3, which contains ester-linkages in both CD moieties and crosslinking domains, exhibited extensive cytoplasmic diffusion of fluorescence, suggesting a loss of structural integrity. Conversely, the fully amide-linked NG1 maintained a more localized and compact fluorescence pattern, indicating higher structural stability against intracellular degradation. The hybrid NG2 system showed intermediate behavior, reflecting the balance between network stability and hydrolytic sensitivity.

Regarding network architecture, NG2 and NG3 display lower swelling ratios than NG1, indicating a higher effective crosslinking density due to the increased incorporation of multifunctional CD moieties. Theoretically, a denser network would be expected to enhance structural stability and slower hydrolysis. However, despite the higher crosslinking density, NG2 and NG3 showed reduced intracellular stability compared to fully amide-linked NG1. This indicates that the degradation kinetics of these systems are primarily governed by the intrinsic hydrolytic sensitivity of the ester bond, rather than physical network architecture. Under mildly acidic intracellular environments, protonation of the ester carbonyl increases its electrophilicity, thereby facilitating nucleophilic attack by water. This process is likely further catalyzed by lysosomal esterases.^{51,59,117} Furthermore, the cleavage of ester bonds within the crosslinking domains directly disrupts network connectivity, thereby accelerating the nGel destabilization. Collectively, these observations indicate that the chemical composition of network, rather than structural differences, serves as the primary molecular “tuner” for modulating intracellular behavior of these CD-nGels.

Notably, a clear difference in C6 fluorescence intensity among the CD-nGel systems was observed only during the early stage of cellular uptake, suggesting a relatively slower initial release from the fully amide-crosslinked NG1 owing to its higher structural stability. By 24 h, however, all three formulations exhibited strong green fluorescence from C6,

indicating substantial release of the encapsulated cargo. This behavior is likely attributed to the relatively low binding constant of the CD/C6 ICs (approximately 218.88 M^{-1}), where dilution alone is sufficient to facilitate near-complete drug release.^{44,100} In contrast, for drugs with significantly higher binding affinities (eg, binding constant $\approx 10,000 \text{ M}^{-1}$), which were not the primary focus of this study, release mechanisms such as competitive displacement, plasma or tissue protein binding, and CD elimination becomes much more critical.⁴⁴ For example, Durk's group found that the strong binding between fenebrutinib and hydroxypropyl- β -CD ($K \approx 2 \times 10^5 \text{ M}^{-1}$) resulted in decreased drug absorption in a CD concentration-dependent manner due to limited release.¹¹⁸ Similar challenges exist for drugs like tamoxifen ($K \approx 0.9\text{--}1.2 \times 10^4 \text{ M}^{-1}$),¹¹⁹ econazole ($K \approx 2.9 \times 10^4 \text{ M}^{-1}$),¹²⁰ and various adamantyl derivatives ($K \approx 10^3\text{--}10^5 \text{ M}^{-1}$),^{121–123} where simple dilution is insufficient to drive efficient drug delivery. In such cases, the chemical cleavage of CD moieties and redistribution of nGels could provide a trigger to facilitate the release of tightly bound complexes. Although this mechanism was not directly explored here, our results demonstrate that our CD-nGel system platform offers a versatile strategy to modulate the degradability and intracellular distribution through rational molecular design.

To translate our findings into more precise therapeutic applications in controlled drug delivery, further systematic studies are required to better understand the relationship between network composition, degradation kinetics, and in vivo therapeutic performance. While our current results highlight the susceptibility of ester-containing CD-nGels to intracellular environments, subsequent studies should aim to clarify the catalytic pathways and mechanism responsible for ester bond cleavage. For example, incorporating enzyme-mediated degradation assays and esterase activity quantification will be essential to identify the specific catalytic contributions to network dissociations. Furthermore, characterizing the metabolic pathways and long-term biocompatibility of degradation byproducts is a prerequisite for clinical transition. It is also critical to acknowledge that nGels internalization is inherently cell-type dependent, pathways can differ across epithelial, macrophage, and distinct cell lines.^{124,125} Expanding this versatile CD-nGel platforms to a broader library of cell lines will be necessary to validate the general applicability of this design strategy, which can provide valuable insights into clinical potential. Finally, future studies will include quantitative evaluation of drug release kinetics using clinically relevant therapeutic agents rather than fluorescent model compounds C6, to better understand the relationship between nGel degradation and delivery behavior. Such study will provide a more rigorous test of whether the tunable degradability within CD moieties can serve as a true on-demand release trigger for drugs used in clinical fields. These efforts could be combined with the further studies of multi-functional, stimuli-responsive drug delivery platforms.^{111,126–129} For instance, recent study in droplet-microfluidic hydrogels have demonstrated the power of precise structural control for simultaneous temperature detection and analysis.¹²⁸ Likewise, Tai et al reported a glucose-responsive nanozyme hydrogel, in which local acidification promoted material degradation and achieved sophisticated therapeutic release.¹²⁹

In summary, this study establishes a versatile design framework for the development of nanomedicine delivery systems with tunable compositions and adjustable intracellular stability. Unlike conventional hydrolytically responsive nGel systems, which typically rely on modifications of polymer backbone or crosslinker chemistry, our approach introduces an additional control by integrating hydrolytically labile linkages directly within the CD host units. In this way, the CD moieties function as dual-purpose motifs: they provide essential host–guest binding sites for hydrophobic pharmaceutical molecules while simultaneously serving as a structural “handle” to modulate the overall hydrolytic behavior. This integrated design enables a more precise control over the intracellular stability and distribution of nGel systems, providing a solid design basis for the future development of programmable “smart” drug delivery systems.

Conclusions

In summary, a series of NB-labeled fluorescent CD-nGels was successfully synthesized via a surfactant-free precipitation polymerization method. The resulting CD-nGels exhibited homogeneous hydrodynamic diameters ranging from 247 nm to 431 nm with Pdl values below 0.2, indicating the monodisperse systems.⁸⁴ By combining two types of modified β CD, amide-linked MA β CD and ester-linked Ac β CD, with crosslinkers of different hydrolytic sensitivities (amide-based BIS and ester-based DEGDA), we obtained three CD-nGel systems (NG1, NG2, and NG3) with progressively enhanced hydrolytic degradability. A key novelty of this work lies in the “dual-handle” tuning of hydrolytic behavior both β CD functionalization and crosslinker chemistry. Unlike most previously reported hydrolytically tunable systems, where stability is primarily regulated through the polymer backbone or crosslinking domains, our platform introduces

degradability directly at the CD host units, thereby providing an additional handle to modulate nGel intracellular behavior. Among three systems, NG3, which incorporated ester linkages in both the β CD moieties and DEGDA crosslinker, exhibited the most pronounced degradation under both mildly acidic (pH 5.1) and intracellular-mimicking MCF-7 lysate conditions, while the fully amide-linked NG1 remained structurally intact. All CD-nGels effectively encapsulated the hydrophobic model drug C6 within the β CD cavities via host–guest interactions. Cytotoxicity assays confirmed their excellent cell compatibility and uptake studies in MCF-7 cells revealed chemistry-dependent intracellular stability and fluorescence distribution patterns of CD-nGels. NG1 maintained a more compact intracellular localization, whereas the ester-containing NG2 and NG3 displayed progressively diffuse intracellular fluorescence patterns, correlating with their reduced structural stability. Overall, this work establishes a modular CD-nGel platform in which intracellular stability and distribution can be tuned through rational chemical design. This dual-handle strategy provides a solid foundation for future studies at developing CD-nGels for controlled intracellular drug delivery.

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Disclosure

Prof. Dr. Patrick van Rijn holds shares in BiomACS, outside the submitted work. The authors report no other conflicts of interest in this work. This paper is based on the thesis of Yanjing Ji. It has been published on the institutional website: <https://research.rug.nl/en/publications/stimuli-responsive-nanogels-for-drug-delivery-and-dental-applicat/>

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