

Renal-Targeted Inulin-Based Gd/Zn Hybrids for Safe MRI Contrast and Early Fibrosis Mitigation

Dandan Wang^{1,2,*}, Wenyi Zhang^{2,*}, Lin Chen^{2,*}, Yunxiao Mai², Haoran Wang², Zhenwei Miao¹, Yang Zhao²

¹Department of Radiology, Tianjin Medical University Baodi Hospital, Tianjin, People's Republic of China; ²Department of Radiology, The Second Hospital of Tianjin Medical University, Tianjin, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yang Zhao, Department of Radiology, The Second Hospital of Tianjin Medical University, Tianjin, People's Republic of China, Email yang.zhao@tmu.edu.cn; Zhenwei Miao, Department of Radiology, Tianjin Medical University Baodi Hospital, Tianjin, People's Republic of China, Email mzwhospital@163.com

Purpose: Gadolinium-based contrast agents (GBCAs), which are indispensable for magnetic resonance imaging (MRI), carry a severe risk of nephrogenic systemic fibrosis (NSF) in patients with chronic kidney disease (CKD), particularly in those with advanced renal impairment. This critical safety concern necessitates the development of biocompatible alternatives that do not compromise diagnostic efficacy.

Materials and Methods: Inulin-based nanoparticles (Inulin@Gd/Zn NPs) were synthesized by chelating inulin with gadolinium and zinc ions, aimed at being used as contrast agents that can replace conventional GBCAs. The morphology, size, and zeta potential of the nanoparticles were determined. The anti-fibrotic ability of Inulin@Gadolinium/zinc nanoparticles (Inulin@Gd/Zn NPs) was evaluated in vitro and in vivo, as well as their MRI imaging ability and metabolic performance in normal and fibrotic kidneys. A unilateral ureteral obstruction (UUO) model was created in mice and was used to evaluate the in vivo anti-fibrotic efficacy.

Results: The ultra-small Inulin@Gd/Zn NPs (<10 nm) exhibited high stability and T1 relaxivity. Compared to Gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA), Inulin@Gd/Zn NPs demonstrated precise kidney targeting, excellent T1-weighted MRI capabilities, and rapid renal clearance. In vitro studies showed that Inulin@Gd/Zn NPs had very low cytotoxicity for HK-2 cells up to 100 μ M. Further, the NPs were able to reduce reactive oxygen species generation in hydrogen peroxide-stimulated HK-2 cells. Negligible levels of hemolysis and organ toxicity were confirmed in vivo. Further, in the UUO model, administration of Inulin@Gd/Zn NPs was found to significantly inhibit fibrosis. Both in vitro and in vivo experiments demonstrated that Inulin@Gd/Zn alleviated GBCA-induced early tubulointerstitial fibrosis by inhibiting the transforming growth factor- β 1 (TGF- β 1) signaling pathway.

Conclusion: Inulin@Gd/Zn NPs represent a promising strategy for clinical application of GBCAs. It addresses the diagnostic and therapeutic challenges in patients with CKD, offering a paradigm shift in the safe application of GBCAs in high-risk populations, indicating the need for further clinical trials to enable clinical applications.

Keywords: renal fibrosis, chronic kidney disease, GBCAs, magnetic resonance imaging

Introduction

Many kinds of kidney problems can be handled with surgery or standard drug therapy, but chronic renal failure is still left with little more than palliative support for chronic kidney disease (CKD).^{1,2} When no true preventive steps or effective medicines are available, the condition tends to move on to end-stage renal disease and then serious cardiovascular complications, and that course often ends in an early death.³ A typical pathologic feature along the way is renal fibrosis, during which extracellular matrix keeps piling up inside the kidneys.^{4,5} Among all of the known pro-fibrotic stimuli, transforming growth factor- β 1 (TGF- β 1) stands out as the major one and its signaling dominates the fibrotic process.⁶ After being released, TGF- β 1 triggers phosphorylation of the type I TGF- β receptor (TGF- β R1); the activated receptor then tweaks the transcription of several fibrotic genes, for instance alpha-smooth muscle actin (α -SMA), so the fibrotic

cascade rolls on.^{7,8} For that reason, getting a clear picture of how TGF- β R1 behaves in the earliest phases of chronic kidney disease is vital for designing treatments that could slow fibrosis and help keep kidney function intact.

Nephrogenic systemic fibrosis (NSF) is a rare, sometimes deadly disorder that can develop after patients with acute kidney injury (AKI) or advanced CKD are given gadolinium-based contrast agents (GBCAs).^{9–12} In the past few years, new rules were brought in to keep NSF from happening. They advise checking renal function ahead of time, choosing a lower-risk GBCA, and cutting the dose whenever possible.¹³ Even so, the rules have sparked an unexpected problem. Critically ill kidney patients who clearly need a contrast-enhanced MRI are now often turned away or have their scan pushed off. Those delays raise the chances of doctors missing the real diagnosis or finding it too late.^{14,15} Although the occurrence of NSF has limited the clinical application of GBCAs, they remain essential for high-resolution MRI because non-gadolinium agents (such as iron oxide) lack the unparalleled relaxivity and versatility of GBCAs.^{16,17} Current strategies focus on macrocyclic GBCAs with higher kinetic stability; however, residual risks persist in patients with end-stage renal disease.^{18–21} In addition to safety, an ideal contrast agent should actively inhibit the progression of CKD, particularly early renal fibrosis, a key ability that is lacking in existing GBCAs.

To promote the biosafety and effective renal clearance of GBCAs, we synthesized novel Inulin@Gadolinium/zinc nanoparticles (Inulin@Gd/Zn NPs) using inulin as a scaffold to chelate gadolinium and zinc ions (Figure 1A). These NPs are intended to be used as GBCAs that can enable good visualization on MRI without posing the risk of renal fibrosis that is associated with conventional GBCAs. Zinc, an essential trace element, exhibits antifibrotic properties by reducing fibroblast activation and extracellular matrix (ECM) accumulation. Therefore, we demonstrate that Inulin@Gd/Zn NPs alleviate renal fibrosis by inhibiting the expression of TGF- β R1/ α -SMA. Inulin is a natural,

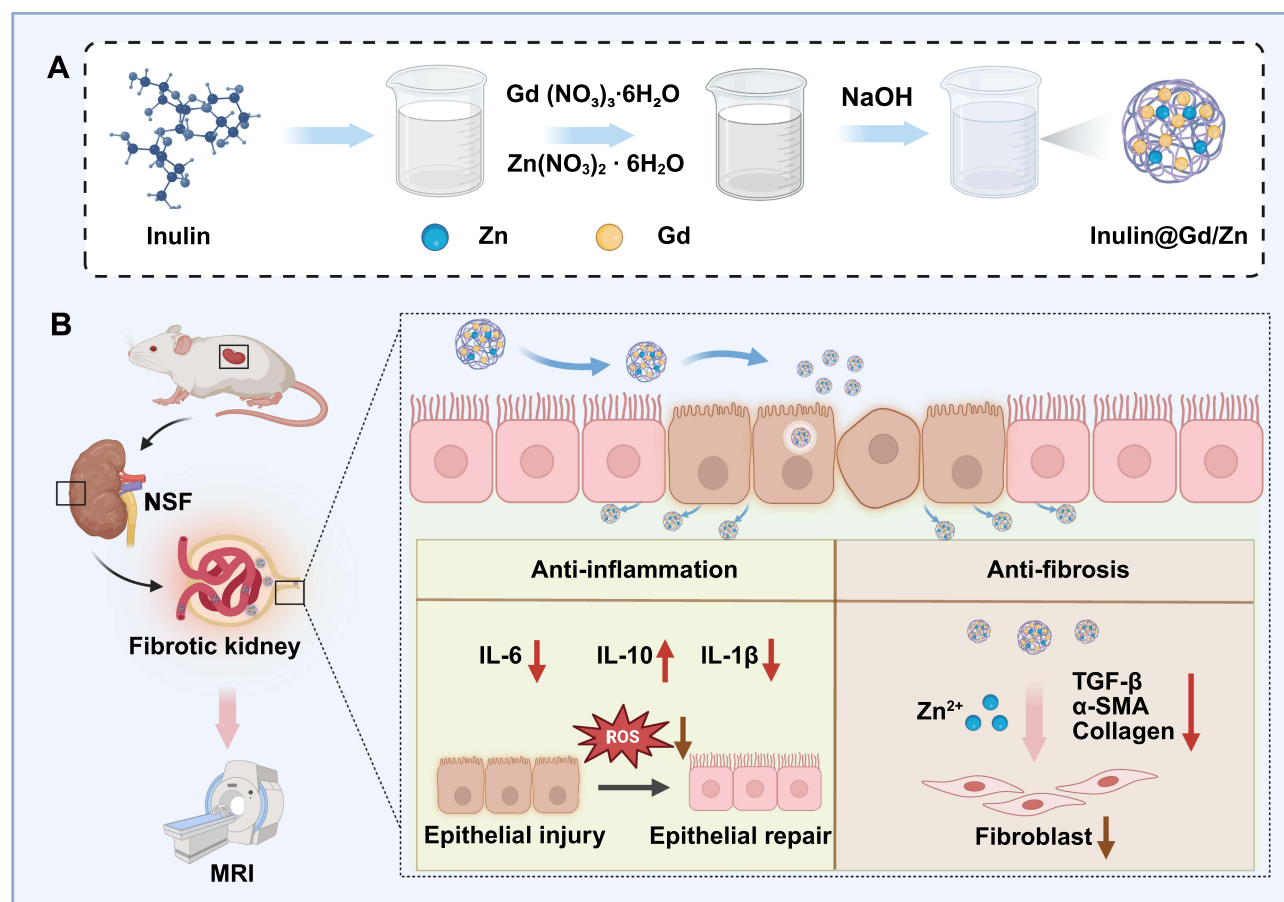


Figure 1 Schematic illustration of Inulin@Gd/Zn NPs for the diagnosis and treatment of mice in UUO models. **(A)** Schematic diagram of the one-pot hydrothermal synthesis process for Inulin@Gd/Zn NPs, which were prepared using inulin, gadolinium nitrate hexahydrate, and zinc nitrate hexahydrate under hydrothermal conditions at pH 8. **(B)** Schematic diagram of the anti-inflammatory and antifibrotic mechanism of Inulin@Gd/Zn NPs in renal fibrosis mice.

biocompatible, and biodegradable polysaccharide composed of linear chains of b-(2-1) fructose units. It possesses amphipathic properties that are attributed to its linear polysaccharide chain structure and abundant hydroxyl functional groups. This structural foundation enables the functionalization of inulin as a versatile nanocarrier that can deliver chemotherapeutic drugs, anti-inflammatory molecules, and nucleic acids to specific lesion sites.^{22–24} Furthermore, inulin comprises hydroxyl groups that can stably coordinate with various metal ions, such as Fe and Zn, either directly or following a chemical modification, facilitating the synthesis of metal-inulin composite nanoparticles.^{25–27} Additionally, owing to its extremely low absorption rate and ultrasmall hydrodynamic size, injected inulin achieves 100% renal filtration, making it the gold standard for assessing renal function. Due to this property of inulin, MRI showed that Inulin@Gd/Zn exhibits kidney-targeted action and rapid clearance dependent on glomerular filtration, thereby reducing gadolinium exposure in the kidneys and throughout the body. In unilateral ureteral obstruction (UUO) models, this study further confirmed that Inulin@Gd/Zn NPs exhibited superior MRI performance, rapid metabolic characteristics, and anti-fibrotic activity compared with Gd-DTPA (Figure 1B). Considering the clinical applications of inulin, Inulin@Gd/Zn NPs may serve as a novel GBCA, opening new directions for therapeutic and diagnostic nanomaterials in precision nephrology.

Materials and Methods

Materials and Chemicals

Human renal human kidney-2 (HK-2) cells (catalog No. SNL-165) and human umbilical vein endothelial cells (HUVECs) (catalog No. SNP-H204) were obtained from SunnCell. Gadolinium nitrate (catalog No. G106605) and zinc nitrate (catalog No. Z111706) came from Aladdin, Shanghai, China. A reactive oxygen species (ROS) assay kit and Fluorescein isothiocyanate (FITC) rapid labeling kit were supplied by Beyotime Biotechnology. Their respective catalog numbers were S0034S and P0639M. The hematoxylin and eosin staining kit (catalog No. G1120), together with the Masson trichrome staining kit (catalog No. G1340), were both purchased from SolarBio in Beijing. The α -smooth muscle actin polyclonal antibody (catalog No. G1340) was from SolarBio as well. The TGF- β 1 monoclonal antibody (catalog No. sc-130348) was purchased from Santa Cruz Biotechnology. The GAPDH monoclonal antibody (catalog No. 60004–1) was from Proteintech. Prestained Protein Marker (catalog No. 26616) was obtained from Servicebio. Cell Counting Kit-8 (CCK-8) was sourced from FineTest (Catalog No. FNCK064).

Preparation of Inulin@Gd/Zn NPs

Inulin@Gd/Zn nanoparticles were synthesized using a biomolecule-templated chelation and co-precipitation method. Briefly, 125 mg inulin was added into 8 mL deionized water and stirred in a water bath at 40 °C for 30 min. Then, 31.14 mg each of gadolinium nitrate and zinc nitrate were separately dissolved in 1 mL each of deionized water. Then, the gadolinium nitrate and zinc nitrate solutions were added in that order to the inulin solution and allowed to react in a water bath at 40 °C for 4 h. At the start, the pH of the solution was adjusted to 8.0 using a 1 mM NaOH solution. Finally, the solution was centrifuged for 10 min at 10000 rpm and ultrafiltration was performed twice for 8 min at 5000 rpm.

Characterization of Inulin@Gd/Zn NPs

X-ray photoelectron spectroscopy (XPS) analysis was performed using an X-ray photoelectron spectrometer (Thermo Fisher IS5). Fourier transform infrared (FTIR) spectra were obtained using a spectrophotometer (Thermo Fisher IS5). Zeta potentials and mean hydrodynamic sizes were assessed using dynamic light scattering (DLS) (Mastersizer 3000, Malvern Panalytical, U.K.). The morphology of the nanoparticles was characterized by transmission electron microscopy (TEM). A total of 100 particles were manually measured, and their size distribution profile was analyzed using ImageJ software.

Stability and Biosafety Evaluation of Inulin@Gd/Zn NPs

Inulin@Gd/Zn nanoparticles were stored at 4°C and their stability was evaluated over 14 days in various media, including phosphate-buffered saline (PBS), saline (0.9% NaCl), RPMI-1640 cell culture medium, and deionized water. Fresh mouse red blood cells were incubated with 37.3 µg/mL Inulin@Gd/Zn at 37°C for 2 h to assess hemolytic activity. PBS and deionized water served as negative and positive controls. Healthy mice were intravenously injected with Inulin@Gd/Zn (Gd 0.1 mmol/kg) via the tail vein. At 24 h post-injection, blood samples were collected for complete blood count (CBC) analysis, including red blood cells (RBC), white blood cells (WBC), hemoglobin (HGB), platelets (PLT), lymphocytes (LYM) and Granulocytes (GRAN). Meanwhile, Major organs (heart, liver, spleen, lungs, and kidneys) were harvested for histopathological examination. The tissues were fixed, sectioned, and stained with hematoxylin and eosin (HE) for microscopic evaluation.

Inulin@Gd/Zn NPs Cell Uptake in vitro

FITC labeled nanoparticles were used to study cellular uptake. FITC was prepared following the manufacturer's instructions. An alkaline buffer (0.1 M NaHCO₃) was added to Inulin@Gd/Zn to adjust the pH value to 8.5. Subsequently, FITC was added to the aforementioned solution under dark conditions until the mixture exhibited a pale yellowish-green hue. The resultant solution was then transferred to a refrigerator at 4 °C and stirred in the dark for 4–6 h. Finally, ultrafiltration was performed three times at a rotational velocity of 4000 rpm to remove unconjugated FITC. HK-2 cells were plated on glass-bottom confocal dishes and allowed to settle overnight. After that, 10 µg/mL FITC-Inulin@Gd/Zn NPs were added and the cultures stayed at 37 °C for 0, 6, 12, or 24 h. The cells were then stained with DAPI (Solarbio, Beijing, China) following the maker's instructions and washed three times with phosphate-buffered saline. Finally, images were collected with a confocal laser scanning microscope, Olympus FluoView FV1000, Tokyo, Japan. Concurrently, flow cytometric assays were conducted on the aforementioned FITC-Inulin@Gd/Zn NPs cells at the matched time points. The flow cytometer was performed using a CytoFLEX S 4L instrument (Beckman Coulter).

In vitro Cytotoxicity of Inulin@Gd/Zn NPs

The cytotoxicity of Inulin@Gd/Zn nanoparticles toward HK-2 cells and HUVECs was examined with the standard CCK-8 assay. In brief, HK-2 cells and HUVECs were cultured in DMEM and DMEM-F12 medium, respectively, supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO₂. HK-2 cells and HUVECs were seeded into each well of a 96-well plate (5000 cells/well) and left overnight so they could settle. The following morning, the medium was switched to fresh medium holding Inulin@Gd/Zn NPs at 100, 50, 25, or 12.5 µM. After a 24 h incubation, we took off the medium and replaced it with serum-free medium mixed with 10% CCK-8, then kept the plate out of direct light for another hour. Finally, absorbance at 450 nm was read on a Varioskan Flash microplate reader (Varioskan Flash, Thermo Scientific).

Reactive Oxygen Species (ROS) Detection in vitro

To find out how Inulin@Gd/Zn NPs clear away ROS, we took 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as the intracellular ROS probe. HK-2 cells were first exposed to 400 µM H₂O₂ for two hours. Afterwards, the cells got several different treatments in the presence of DCFH-DA and were left in the incubator for another 24 h. Following washing, the cells were treated with DCFH-DA for one hour. At the end, fluorescence intensity was read on a confocal laser scanning microscope (FluoView FV1000, Olympus, Tokyo, Japan).

Assessment of the Anti-Inflammatory Effects of Inulin@Gd/Zn NPs

Bone marrow-derived macrophages (BMDMs) were generated by first preparing a single-cell suspension from murine tibiae and femora. The cells were then differentiated for 7 d in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 20 ng mL⁻¹ recombinant mouse macrophage colony-stimulating factor (M-CSF). To assess immunomodulatory effects, differentiated BMDMs were treated for 24 h with PBS, Gd-DTPA, Inulin@Gd/Zn NPs, or Gadobutrol. A negative control group was established by treating the cells with 100 ng mL⁻¹ lipopolysaccharide (LPS). Following treatments, the culture

supernatants were collected to quantify IL-6 and IL-10 expression levels using enzyme-linked immunosorbent assay (ELISA). Concurrently, the BMDMs were harvested for flow cytometric analysis to evaluate M2 polarization. Briefly, the cells were digested to obtain a single-cell suspension and then centrifuged at 1800 rpm for 5 min. The resulting cell pellet was washed and resuspended in PBS containing 1% FBS. Subsequently, the cells were stained for 1 h with the following fluorescence-conjugated antibodies (all purchased from BioLegend, San Diego, CA, USA): anti-mouse PE/Cy7-CD11b (Cat# 101216), anti-mouse FITC-F4/80 (Cat#123107), and anti-mouse PE-CD206 (Cat# 1141706). Following staining, the cells were washed again, centrifuged, and finally resuspended in PBS with 1% FBS for acquisition on a flow cytometer.

Western Blotting

Protein expression was examined by Western blotting in HK-2 cells that had received different treatments. First, the cells were exposed to 10 ng mL^{-1} TGF- β 1 for 24 h.²⁸ They were then given the same volume of PBS, Gd-DTPA, Gadobutrol or Inulin@Gd/Zn (Gd 50 μM) and kept for another 24 h. After removing the medium, the monolayer was washed twice with ice-cold PBS, and the pellet was resuspended in chilled radioimmunoprecipitation assay (RIPA) lysis buffer. The mixture was pipetted until no clumps were seen, followed by centrifugation at 4 °C for 15 min; the clear supernatant was saved for later use. Whole-cell extracts intended for denaturing gels were boiled in sodium dodecyl sulfate (SDS) sample buffer at 99 °C for 10 min. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to a polyvinylidene difluoride (PVDF) membrane with a Bio-Rad transfer unit, and straightaway blocked in 5% skim milk made up in tris-buffered saline (TBS) containing 0.1% Tween 20 (50 mM Tris, pH 7.6, 150 mM NaCl). The blots were rinsed for five minutes in TBS-Tween and incubated overnight at 4 °C with the primary antibody (TGF- β 1 monoclonal antibody, catalog No. sc-130348, Santa Cruz Biotechnology; Smad 3 monoclonal antibody catalog No.sc-534535, Santa Cruz Biotechnology and GAPDH monoclonal antibody, catalog No. 60004–1, Proteintech). The next day, they were treated with another HRP-conjugated anti-mouse antibody for one hour at room temperature. Bands were visualized using an ECL chemiluminescent substrate.

Scratch Assay

Cells were plated in six-well plates and grown in DMEM-F12 containing 10% FBS until a confluent monolayer formed. The medium was removed, and a straight scratch was drawn through the cell layer with a 200 μL pipette tip. Baseline images were taken immediately on the microscope. Cultures were then kept for 24 hours in fresh medium that included 10 ng/mL TGF- β 1. After that incubation, either Gd-DTPA or Inulin@Gd/Zn or Gadobutrol was added so the final Gd concentration reached 50 μM . A second set of pictures was collected. Scratch width was determined with ImageJ, and the data were normalized before analysis.

Model Animals

This study was approved by The Second Hospital of Tianjin Medical University, Tianjin, China. All animal experiments were reviewed and approved by the institutional animal care and use committees of Tianjin University of Traditional Chinese Medicine (Approval No. TCM-LAEC2023103), in accordance with the Guidelines for the Ethical Review of Laboratory Animal Welfare (GB/T 35892–2018) for the care and use of animals.

Unilateral Ureteral Obstruction (UUO) Model Creation and Treatment

A unilateral ureteral obstruction model was set up in 6-to-8-week-old C57BL/6 mice. In short, each mouse was put under general anesthesia with sodium pentobarbital at 50 mg per kg by intravenous injection, then a small cut was made along the midline of the abdomen. The left ureter was located and tied twice with 4–0 silk thread. The incision was closed with stitches, and after the animals woke up and showed no unusual signs, they were placed back in their cages. For sham-operated mice, the ureter was only exposed without being tied. The animals were randomly divided into four groups: (1) Control: Sham operation; (2) UUO: UUO operation for 7 and 14 days; (3) Gd-DTPA group: Gd-DTPA was administered via tail vein injection on days 3, 5, and 7 after UUO surgery; and (4) Inulin@Gd/Zn NPs group: Inulin@Gd/Zn NPs were administered via tail vein injection on days 3, 5, and 7 after UUO surgery. On days 7 and 14, mice were euthanized for subsequent efficacy evaluations, including histological and molecular analyses.

Relaxivity Measurement and in vivo MRI of Inulin@Gd/Zn NPs

The longitudinal relaxivity of Inulin@Gd/Zn, Gd-DTPA and Gadobutrol was determined by measuring the T_1 relaxation times at varying Gd concentrations using a 3.0 T MRI scanner. The r_1 relaxivity ($1/s^{-1}$) was obtained from the slope of the linear regression of $1/T_1$ versus Gd concentration. SO and UUO mice were intravenously injected via the tail vein with Inulin@Gd/Zn or Gd-DTPA at a dose of 0.1 mmol Gd/kg. T_1 -weighted MR images were acquired before injection (baseline) and at 5 min, 30 min, 1 h, 2 h, 3 h, 6 h, and 12 h post-injection. The signal intensities in regions of interest (ROIs) were quantified, and time-dependent enhancement curves were plotted to compare the dynamic contrast behaviors of the two agents in different kidney conditions. To further investigate the in vivo biodistribution of Gd, healthy mice were randomly divided into two groups ($n=3$) and received tail vein injection of Inulin@Gd/Zn NPs and Gadobutrol. On days 1 and 3 post-injection, major organs, namely the heart, liver, spleen, lungs, kidneys, and brain, were harvested, and Gd content in each organ was quantified using inductively coupled plasma mass spectrometry (ICP-MS).

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Kidney samples from the control, PBS, Gd-DTPA and Inulin@Gd/Zn NPs groups were collected on the 7th and 14th day after surgery, then were quick frozen in liquid nitrogen and stored at -80°C . Total mRNA was isolated with TRIzol reagent. Next, complementary DNA (cDNA) was synthesized with the commercial kit supplied by Cowin Biotech (China) according to the instructions that came with it. Finally, the relative messenger RNA (mRNA) level of each target gene was assessed by real-time quantitative PCR. The primer sets were as follows:

mIL-1 β forward, 5'-GAAATGCCACCTTTTGACAGTG-3' and reverse, 5'-TGGATGCTCTCATCAGGACAG-3'; mIL-6 forward, 5'-TAGTCCTTCCTACCCCAATTTC-3' and reverse, 5'-TTGGTCCTTAGCCACTCCTTC-3'; mTGF β forward, 5'-GCTGCAGGCCTTTGATGTG-3' and reverse, 5'-TTGAGAAGGACTGCCACGAC-3'; mTNF α forward, 5'-CCCTCACACTCAGATCATCTTCT-3' and reverse, 5'-GCTACGACGTGGGCTACAG-3'; mGAPDH forward, 5'-GACAGCCGCATCTTCTTG-3' and reverse, 5'-AATCCGTTACACCGACCTT-3'.

Masson Staining

The kidney samples were first fixed in formalin, then put into paraffin and sliced into roughly 5-micron sections for slides. Once the paraffin was taken off, the sections were treated with a Masson staining kit. Finally, we looked at the stained slides with a microscope.

Immunohistochemical Staining

Kidney samples were fixed in neutral formalin, embedded in paraffin wax, and cut into five micron thick sections. After the wax was removed, antigen retrieval was carried out and the slides were allowed to cool at room temperature. To block the natural peroxidase, the sections sat in 0.3% hydrogen peroxide dissolved in methanol for twenty minutes. They were then placed in five percent BSA for about an hour to finish the blocking step. Following three quick rinses with PBS, the sections were incubated overnight at 4°C with the primary antibody α -SMA. Subsequent to washing, the slices were incubated at 37°C for 60 minutes with a secondary antibody (HRP-conjugated goat anti-rabbit IgG, DAKO). In the last stage, the slides were dipped in DAB working solution for roughly five minutes, counterstained with hematoxylin, and finally viewed under a light microscope.

Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical differences were analyzed using Student's t -test or one-way ANOVA. $p < 0.05$ or $p < 0.01$ was considered statistically significant or very significant, respectively.

Results and Discussion

Synthesis and Characterization of Inulin@Gd/Zn NPs

We first evaluated the hydrodynamic diameters of Inulin@Gd/Zn NPs with different Gd/Zn ratios. At Gd/Zn ratios between 1:1 and 4:1, the nanoparticles exhibited small particle sizes ([Supplementary Figure S1](#)), with the corresponding drug loading presented in [Table S1](#). Based on a comprehensive consideration of the roles of Gd and Zn in the imaging

process, Gd offers excellent imaging efficacy but carries the risk of inducing NSF. In contrast, Zn supplementation can inhibit the regulation of the TGF- β protein transduction pathway and down-regulate fibrosis factors,^{29,30} therefore, we used the 1:1 ratio to synthesize Inulin@Gd/Zn NPs. Inulin@Gd/Zn NPs were stained with 1% phosphotungstic acid and observed under a TEM (Figure 2A). The images reveal spherical nanostructures with an average size of 7.5 nm (Figure 2D), which is below the upper limit of 10 nm—a requirement for renal excretion.³¹ The elemental mapping of the Inulin@Gd/Zn NPs confirmed the presence of C, O, Zn, and Gd (Figure 2B and C). DLS analysis indicated that the average size of the Inulin@Gd/Zn NPs was 8.7 nm, and the average Zeta potential was found to be 0.29 ± 0.022 mV (Supplementary Figure S2). FTIR spectroscopy of inulin and Inulin@Gd/Zn NPs (Figure 2E) was performed, and the changes in the spectral peaks suggested the successful synthesis of the product, which aligns with earlier findings.²⁷ Additionally, XPS was used to characterize the elemental and valence compositions of the Inulin@Gd/Zn NPs (Figure 2F). The Gd4d spectrum confirmed the uptake and partial conversion of Gd4d_{3/2} to Gd4d_{5/2}, with signals detected at 152 and 144 eV representing Gd4d_{3/2} and Gd4d_{5/2}, respectively (Figure 2G). The observed binding energies were consistent with those of gadolinium in the +3 oxidation state. The Zn2p spectrum confirmed the uptake and partial conversion of Zn2p_{1/2} to Zn2p_{3/2}, with signals detected at 1043 and 1020 eV representing Zn2p_{1/2} and Zn2p_{3/2}, respectively (Figure 2H). The observed binding energies were consistent with those of zinc in the +2 oxidation state. To

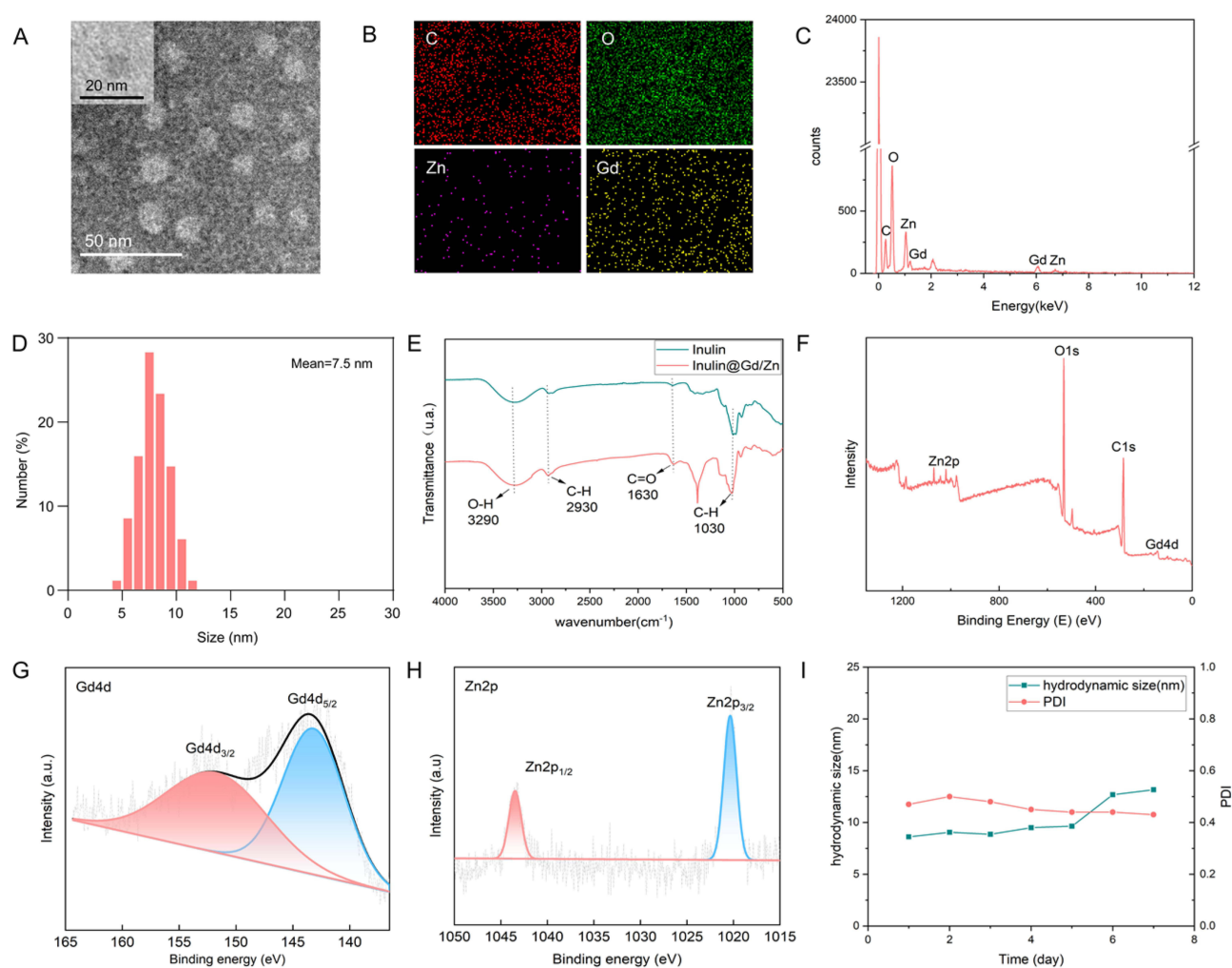


Figure 2 Synthesis and characterization of Inulin@Gd/Zn NPs. (A) TEM image of Inulin@Gd/Zn NPs. Scale bar, 50 nm. (B) Elemental mapping of Inulin@Gd/Zn NPs showing the presence of C, O, Zn, and Gd. (C) X-ray photoelectron spectroscopy (XPS) showing the components of Inulin@Gd/Zn NPs. (D) Size distribution of Inulin@Gd/Zn NPs, as observed using TEM. (E) FT-IR of inulin and Inulin@Gd/Zn NPs. (F–H) XPS peaks of Inulin@Gd/Zn NPs and Gd 4d and Zn 2p peaks. (I) Hydrodynamic size and polydispersity index (PDI) of Inulin@Gd/Zn NPs measured over 7 days.

evaluate the stability of the Inulin@Gd/Zn NPs, their hydrodynamic size and polydispersity index (PDI) were measured from days 1 to 7. [Figure 2I](#) shows that the synthesized inulin@Gd/Zn NPs exhibited good stability and dispersion.

Therefore, we have successfully synthesized this new contrast agent, Inulin@Gd/Zn NPs. Compared to gadolinium-based contrast agents, gadolinium-free contrast agents have also made significant breakthroughs in recent years, including nanodiamonds and metal-based contrast agents containing iron or manganese. The principal advantage of these contrast agents is their capacity to circumvent the potential safety hazards related to the long-term deposition of free gadolinium ions in the body. Nevertheless, the existing gadolinium-free contrast agents still encounter several constraints. Their imaging performance is subpar compared to that of gadolinium-based contrast agents, with a feeble signal enhancement ability in T1-weighted imaging. Iron oxide nanoparticles are utilized for T2 imaging but are prone to T2* signal interference. Additionally, the corresponding imaging techniques are not yet fully developed, and clinical validation is inadequate. Furthermore, their long-term biosafety in the human body awaits systematic assessment.^{32–34} In light of this, traditional gadolinium was still employed for MRI in our study. Gadolinium-based contrast agents present notable advantages in T1 relaxation time, producing brighter positive contrast enhancement effects, and their image quality surpasses that of contrast agents containing other metals.³⁵ Moreover, we introduced Zn into the agent, aiming to mitigate the risk of NSF induced by gadolinium ions. Thus, while maintaining the excellent imaging performance of gadolinium-based agents, their safety characteristics are further improved.

Cellular Uptake and Cytotoxicity of Inulin@Gd/Zn NPs

The cellular uptake of Inulin@Gd/Zn NPs was confirmed using *in vitro* fluorescence imaging. HK-2 cells were incubated with Inulin@Gd/Zn for 0, 6, 12, or 24 h, and subsequently observed using confocal microscopy. The cellular uptake of Inulin@Gd/Zn NPs by HK-2 cells exhibited a time-dependent trend, with the highest uptake observed at 24 h ([Figure 3A](#)). In addition, to evaluate the uptake kinetics, the uptake of the materials by HK2 cells was quantified at different time points (6, 12, and 24 h) using flow cytometry. The cellular uptake exhibited a significant time-dependent manner, with the uptake rate gradually increasing over time ([Supplementary Figure S3](#)). Before performing further studies, CCK-8 assays were conducted to evaluate the cytotoxicity of Inulin@Gd/Zn NPs in HK-2 and HUVEC cells. Inulin@Gd/Zn NPs exhibited negligible cytotoxicity at concentrations up to 100 μ M, indicating their excellent biocompatibility and safety ([Figure 3B and C](#)).

Inulin@Gd/Zn NPs Can Prevent ROS Production, Anti-Inflammation and Inhibit TGF- β 1

AKI occurs in blocked kidneys during the early stages of ureteral obstruction. This process promotes the production of both inflammatory and fibrotic mediators while simultaneously disrupting mitochondrial function and increasing oxidative stress, thereby elevating ROS levels. Ultimately, this leads to renal tubulointerstitial fibrosis.^{36–38} Removing excess ROS and inhibiting TGF- β 1 signaling have been proven to effectively alleviate renal fibrosis,^{39,40} which is consistent with the functions of Inulin@Gd/Zn developed in this study. To investigate the ROS-scavenging capability of Inulin@Gd/Zn, we employed the DCFH-DA probe to measure intracellular ROS levels. H₂O₂-stimulated HK-2 cells produced substantial amounts of ROS. However, treatment with Inulin@Gd/Zn NPs significantly reduced the ROS production in H₂O₂-stimulated HK-2 cells ([Figure 3D](#)).

The anti-inflammatory effects of Inulin@Gd/Zn NPs, Gd-DTPA, and Gadobutrol were validated *in vitro* using mouse BMDMs. Treatment with inulin significantly promoted M2 macrophage polarization, with M2 macrophages accounting for approximately 8% of the total cell population. Notably, this proportion was four times higher than that induced by Gd-DTPA, indicating the potent anti-inflammatory capacity of Inulin@Gd/Zn NPs ([Supplementary Figure S4](#)). Cytokine profiling using ELISA demonstrated that Inulin@Gd/Zn NPs, Gd-DTPA, and Gadobutrol exerted opposing immunomodulatory effects on BMDMs. Specifically, Inulin@Gd/Zn NPs promoted an anti-inflammatory profile by significantly promoting IL-10 and inhibiting IL-6 secretion. In contrast, Gd-DTPA induced a pro-inflammatory shift, characterized by decreased IL-10 and increased IL-6 levels ([Figure 3E and F](#)). Collectively, these results demonstrate the superior anti-inflammatory efficacy of Inulin@Gd/Zn NPs compared to conventional gadolinium-based agents.

[Figure 3G–I](#) and [Supplementary Figure S5](#) demonstrate that Inulin@Gd/Zn NPs significantly delay migration of TGF- β 1 treated HK-2 cells. Gadobutrol only slightly inhibited the migration of TGF- β 1 treated HK-2 cells. However,

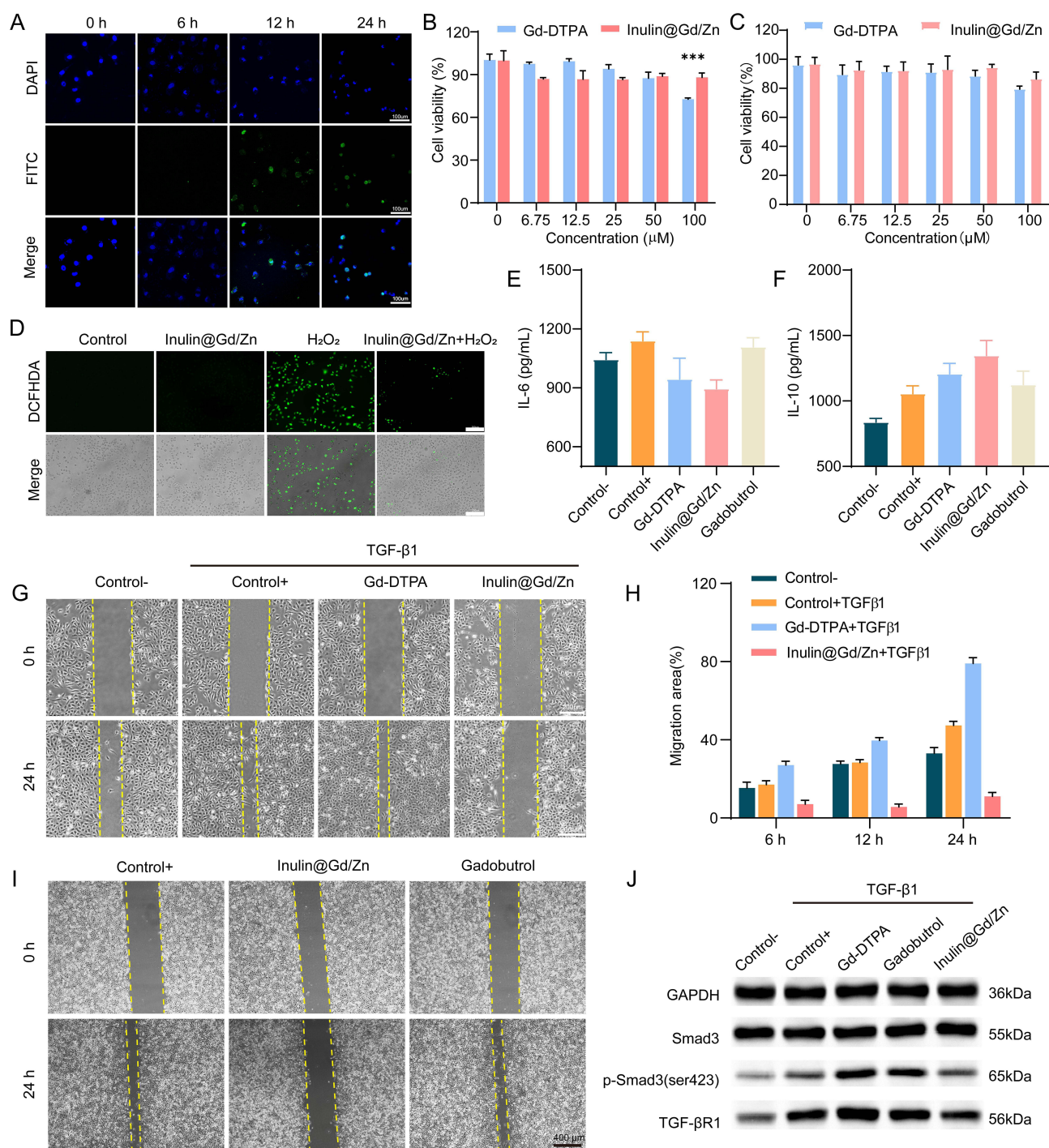


Figure 3 Inulin @Gd/Zn Np cellular uptake, cytotoxicity, and ability to inhibit ROS formation, anti-inflammation and TGF- β 1 expression. **(A)** Microscopy images at different time points of live HK-2 cells cocultured with FITC- Inulin@Gd/Zn NPs. Scale bar, 100 μ m. **(B and C)** Cell viability after treatment with different concentrations of Gd-DTPA and inulin@Gd/Zn NPs in HK-2 cells and HUVECs ($n=3$, *** $p < 0.001$). **(D)** Representative fluorescence microscopy images of HK-2 cells stained with DCFH-DA. Scale bar, 200 μ m. **(E and F)** Levels of IL-6 and IL-10 in macrophage supernatants were determined by ELISA ($n=3$). **(G)** Images of the scratch assay obtained using an optical camera showing cell migration after various treatments for 24 h. The group that was treated only with TGF- β 1 was used as the positive control (Yellow dotted lines represent the scratch assay). **(H)** Quantitative analyses based on scratch assay shown in **(G)** ($n=3$). **(I)** Images of the scratch assay obtained using an optical camera showing cell migration in different treatment groups after TGF- β induction for 24 h. **(J)** Western blotting showing the expression of TGF- β R1 and Smad3 after various treatments.

Gd-DTPA promoted HK-2 cell migration, indicating that Gd promotes renal fibrosis. These findings confirmed the superiority of Inulin@Gd/Zn NPs in inhibiting the progression of renal fibrosis. A pivotal characteristic of fibrosis is the transformation of overactivated fibroblasts into myofibroblasts, which further leads to the excessive accumulation of

extracellular matrix and thereby accelerates fibrosis progression. In this pathological process, TGF- β 1 plays a central regulatory role. Alterations in cell migratory capacity can serve as a biological marker for assessing the progression of fibrosis, and the cell scratch assay is a commonly used method in cell biology for evaluating cell migratory ability.⁴¹

AKI promotes progression of CKD and renal fibrosis, which are inevitable consequences of almost all forms of progressive CKD. As stated above, TGF- β 1 signaling serves as the master regulator of the fibrosis pathway, which, along with its downstream signaling pathways, is considered a critical therapeutic target for renal fibrosis.^{42,43} Therefore, we investigated whether Inulin@Gd/Zn NPs could inhibit TGF- β 1 signaling. Western blot analysis revealed that exposure to TGF- β 1 upregulated TGF- β 1R and its downstream Smad3 signaling pathway expression, particularly in the Gd-DTPA group, whereas treatment with Inulin@Gd/Zn NPs significantly attenuated TGF- β 1R and Smad3 expression (Figure 3J), suggesting that Inulin@Gd/Zn can suppress renal fibrosis. The quantitative diagrams of the corresponding proteins are presented in the [Supplementary Figure S6](#).

These findings demonstrate that Inulin@Gd/Zn NPs can inhibit GBCAs-induced ROS generation, reduce inflammation, and downregulate TGF- β 1 expression in vitro. Its core mechanism of action is attributed to the integration of Zn within Inulin@Gd/Zn NPs. Evidence shows that Zn²⁺ exerts a regulatory influence on the pathological progression of renal fibrosis. Zn²⁺ could directly bind to the zinc finger domain of nuclear factor-kappa B (NF- κ B), thereby impeding its nuclear translocation. This inhibition consequently suppresses the release of pro-inflammatory factors, such as TNF- α and IL-6, thus alleviating the local inflammatory microenvironment in the kidney. Simultaneously, by inhibiting the TGF- β /Smad2/3 pathway, Zn²⁺ reduces collagen deposition and blocks inflammation-driven fibrosis initiation. Additionally, zinc serves as an essential cofactor for various antioxidant enzymes, such as superoxide dismutase. It stabilizes their active sites and enhances their ROS scavenging capacity, thereby mitigating oxidative stress-induced damage to renal cells and further suppressing ROS-mediated fibrotic signaling activation.^{44,45}

MRI Performance of Inulin@Gd/Zn NPs

The MRI performance of the Inulin@Gd/Zn NPs was assessed using a clinical 3.0 T MRI scanner, with Gd-DTPA and Gadobutrol serving as the clinical reference contrast agent. T1-weighted imaging revealed concentration-dependent signal enhancement for Inulin@Gd/Zn NPs, Gd-DTPA and Gadobutrol, with the former exhibiting superior imaging capability (Figure 4A and B).

Subsequent in vivo evaluation of sham-operated (SO) mice showed that Inulin@Gd/Zn produced significantly brighter T1-weighted images than Gd-DTPA. In contrast, Inulin@Gd/Zn NPs significantly enhanced T1-weighted imaging compared with Gd-DTPA, reaching peak contrast enhancement at 30 minutes to 1 h post-injection (Figure 4C and E). The UUO model offers advantages, such as operational simplicity, strong reproducibility, and rapid induction of tubular injury.⁴⁶ Therefore, UUO is frequently employed to create a mouse kidney fibrosis model. In UUO mice, Inulin@Gd/Zn NPs significantly enhanced T1-weighted imaging compared with Gd-DTPA and peaked at 30 min after injection (Figure 4D and F). The enhanced performance of Inulin@Gd/Zn NPs can be attributed to the unique properties of inulin, which is an ideal agent for the determination of glomerular filtration rate (GFR) owing to its small molecular size that facilitates complete glomerular filtration. Given these characteristics, inulin naturally functions as a kidney-targeting delivery vehicle.⁴⁷ Consequently, Inulin@Gd/Zn NPs exhibited significantly faster renal elimination kinetics than Gd-DTPA, thereby minimizing the risk of nephrogenic systemic fibrosis.

Additionally, bladder MRI confirmed efficient renal excretion, with Inulin@Gd/Zn NPs detected in the bladder within 30 min and being completely eliminated within 24 h ([Supplementary Figure S7](#)). Furthermore, quantitative analysis via ICP-MS demonstrated that Inulin@Gd/Zn NPs were predominantly taken up and excreted via the kidneys, with only a trace amount remaining in the body at 72 h post-injection ([Supplementary Figure S8A](#)). In contrast, Gadobutrol was eliminated through both hepatic and renal pathways, and its residual levels in the heart, liver, kidneys, and brain at 72 h post-injection were significantly higher than those of Inulin@Gd/Zn NPs ([Supplementary Figure S8B](#)). These findings underscore the superior MRI performance and favorable pharmacokinetic profile of Inulin@Gd/Zn as a potential contrast agent.

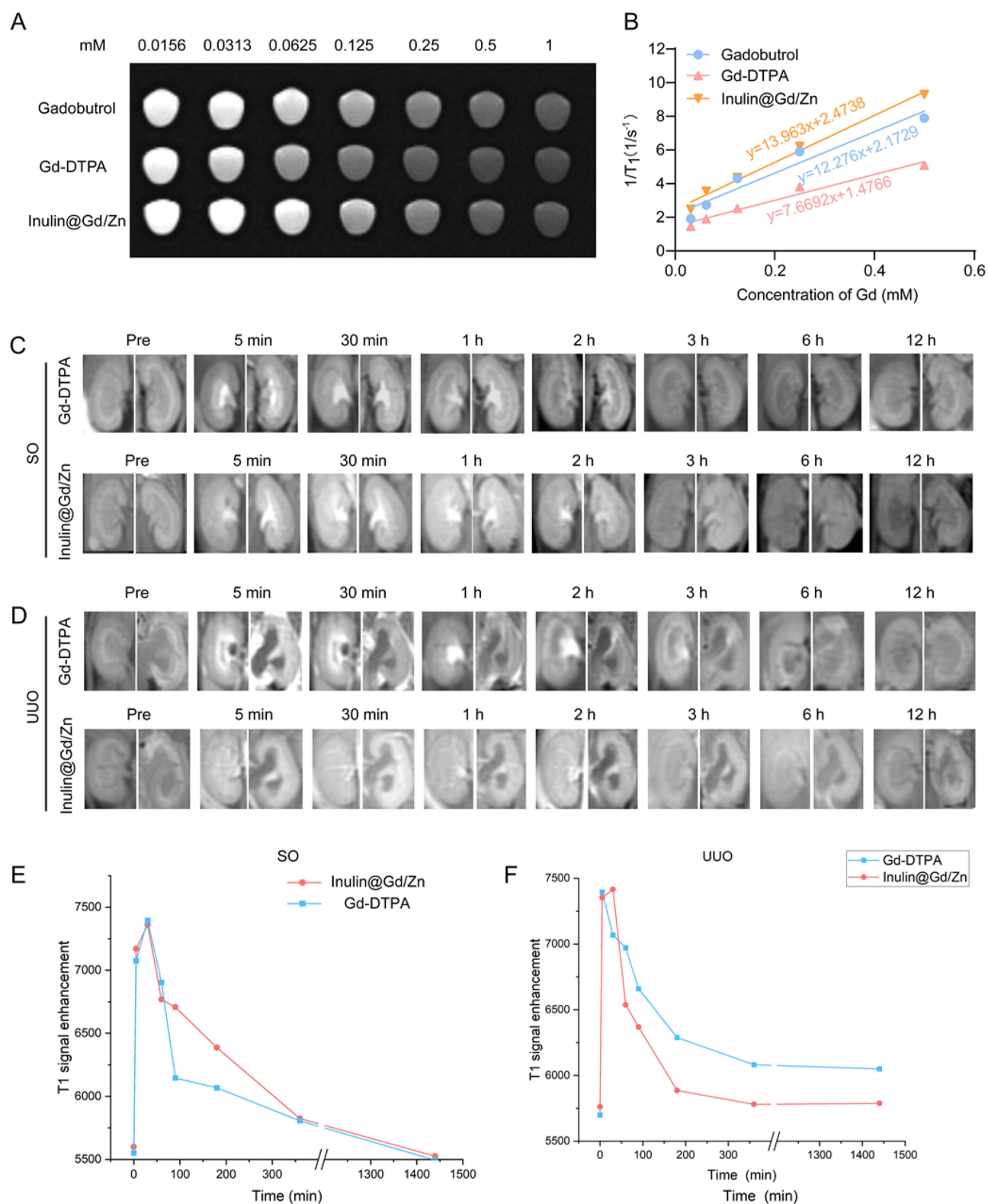


Figure 4 MRI of Gd-DTPA and Inulin@Gd/Zn nanoparticles in sham-operated (SO) and unilateral ureteral obstruction (UUO) mice. (**A** and **B**) T1-weighted in vitro imaging of different concentrations of Gd-DTPA and Inulin@Gd/Zn nanoparticles. Analysis of the longitudinal relaxation rates r_1 ($1/T_1$) of Inulin@Gd/Zn NPs and Gd-DTPA. T1 relativity was calculated from the slopes of the best-fit lines of the experimental data. (**C** and **D**) T1-weighted coronal images of kidneys in SO and UUO mouse models before and at different time points after injection of Gd-DTPA and Inulin@Gd/Zn. (**E** and **F**) Quantitative analyses of imaging ability based on (**C** and **D**).

Evaluation to the Anti-Renal Fibrosis Efficacy of Inulin@Gd/Zn NPs

To determine the optimal treatment duration, we investigated the degree of renal fibrosis on days 7, 14, and 21 after UUO. [Supplementary Figure S9](#) shows the appearance of renal fibrosis on day 7. This corresponds with earlier reports on the pathological features of renal fibrosis in the UUO model.⁴⁸ Thus, therapeutic intervention commenced on post-UUO day 3, with subsequent dosing on days 5 and 7 to inhibit early fibrotic progression ([Figure 5A](#)). This strategy has been widely used in previous studies.^{49,50} The mice were euthanized on days 7 and 14 and peripheral blood and organs were collected.

On days 7 and 14 of treatment, we dissected mice from each group, captured images of their bilateral kidneys, and collected blood for biochemical analyses ([Figure 5B](#)). Creatinine (CREA) and blood urinary nitrogen (BUN) are important indicators of kidney function. In the PBS group, UUO surgery induced severe renal injury in mice, leading to significant glomerular dysfunction and markedly elevated levels of renal injury markers ([Figure 5C and D](#)). Furthermore, Gd-DTPA exacerbated renal fibrosis, particularly in mice with pre-existing severe kidney disease, resulting in further deterioration of renal function. Consequently, the Gd-DTPA-treated group exhibited higher levels of renal injury markers than the PBS group. In contrast, treatment with Inulin@Gd/Zn NPs, which contain zinc and inulin, significantly attenuated renal fibrosis and exerted anti-inflammatory effects, thereby counteracting Gd-DTPA-induced nephrotoxicity and restoring renal function to near-normal levels, as evidenced by the normalized levels of renal injury markers. Masson staining and α -SMA histological immunohistochemistry analysis confirmed that Gd-DTPA aggravated renal fibrosis, whereas Inulin@Gd/Zn NPs treatment effectively mitigated fibrotic progression ([Figure 5E and G](#)). Quantitative data from [Figure 5F and H](#) further supported these findings, demonstrating a significant reduction in collagen deposition and α -SMA expression in the Inulin@Gd/Zn group compared with that in the Gd-DTPA treated group. H&E staining revealed that the Gd-DTPA-treated group showed increased inflammatory cell infiltration. In contrast, Inulin@Gd/Zn NPs treatment significantly attenuated the inflammatory cell recruitment, supporting its anti-inflammatory properties ([Supplementary Figure S10](#)).

Quantitative PCR (qPCR) analysis of renal tissues revealed significantly elevated mRNA expression levels of TGF- β , interleukin 6 (IL-6), interleukin 1 β (IL-1 β), and tumor necrosis factor α (TNF- α) in the Gd-DTPA-treated group, indicating that Gd exacerbates renal fibrosis and inflammatory responses ([Figure 5I–L](#)). In contrast, Inulin@Gd/Zn NPs treatment markedly downregulated these profibrotic and proinflammatory cytokines, demonstrating that Inulin@Gd/Zn NPs effectively counteracted Gd-induced nephrotoxicity and contributed to partial renal functional recovery. These findings suggested that Inulin@Gd/Zn NPs mitigate renal fibrosis and exert potent anti-inflammatory effects in vivo, further supporting their therapeutic potential in preventing Gd-aggravated kidney injury. These data align with the histological observations, reinforcing the conclusion that Inulin@Gd/Zn NPs protect against Gd-induced renal damage by suppressing fibrotic and inflammatory pathways.

This study highlights the protective role of zinc- and inulin-loaded Inulin@Gd/Zn NPs against Gd-induced renal fibrosis and functional impairment in UUO mice, suggesting their potential therapeutic application in mitigating contrast-associated nephropathy. This effect mirrors the mechanism by which Zn mitigates renal fibrosis that was reported by Xu et al.⁵¹

Biosafety Evaluation of Inulin@Gd/Zn NPs

To evaluate the potential hemolytic effects of Inulin@Gd/Zn NPs, we first conducted an in vitro hemolysis assay by spectrophotometrically measuring hemoglobin release following exposure to varying concentrations of the nanoparticles. PBS and pure water (H₂O) served as negative and positive controls, respectively. Inulin@Gd/Zn NPs did not induce significant hemolysis in the red blood cells ([Figure 6A and B](#)). Subsequently, we assessed the in vivo biosafety of Inulin@Gd/Zn. Healthy mice were administered either PBS or Inulin@Gd/Zn and blood was collected daily. At the end of the observation period, the mice were euthanized and blood samples were collected for routine blood analysis. Hematological parameters revealed no significant differences between the control and NP-treated groups ([Figure 6C–H](#)).

To investigate the NP stability, Inulin@Gd/Zn NPs were dispersed in PBS, saline, RPMI-1640 medium, and water, and its stability was monitored over 14 days. The results confirmed the excellent stability of Inulin@Gd/Zn NPs under

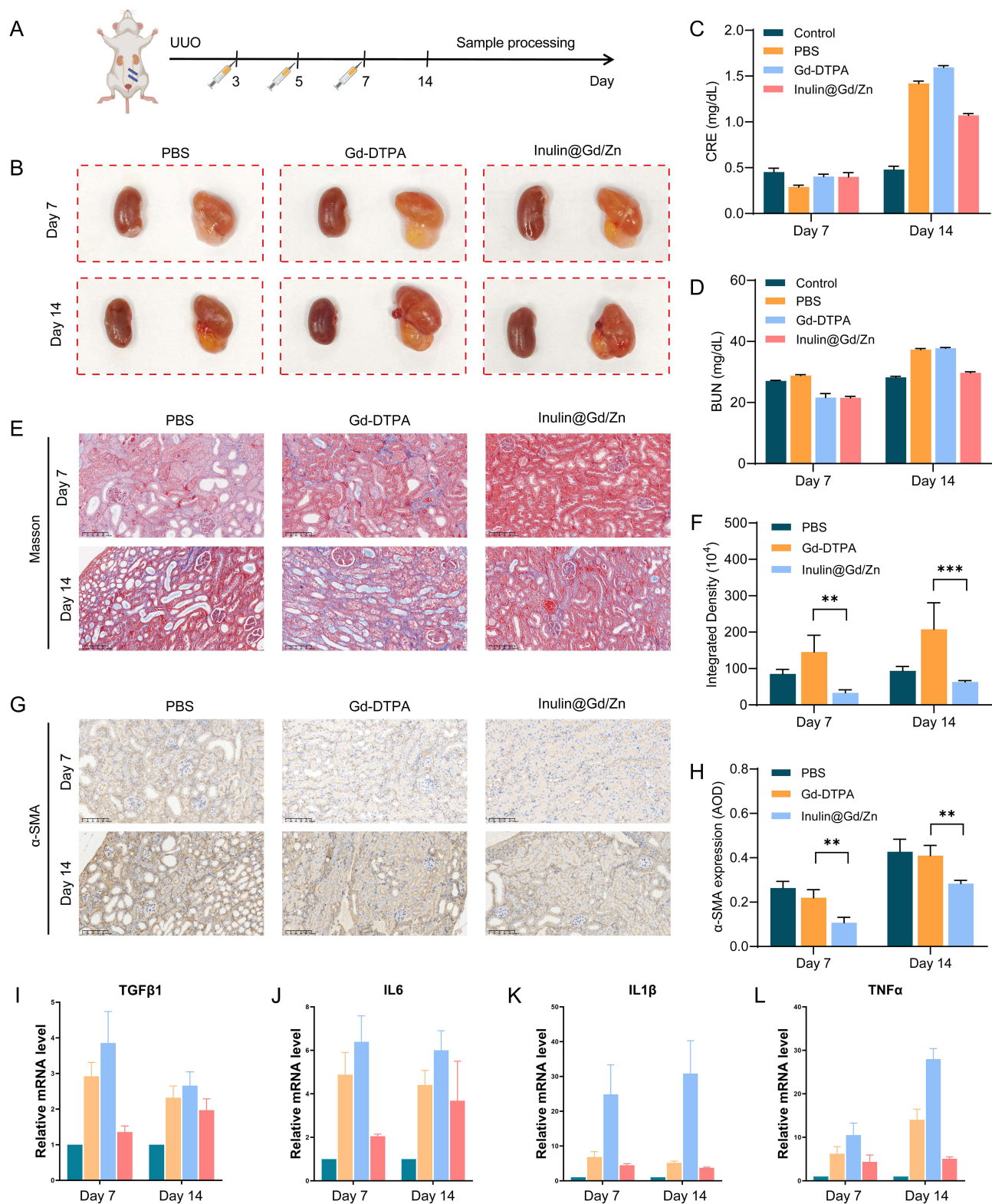


Figure 5 Anti-renal fibrosis efficacy of Inulin@Gd/Zn NPs in vivo. **(A)** The treatment schedule for mice (n=3). **(B)** Representative photographs of kidneys at the end of treatment. **(C and D)** Graphs showing levels of biochemical indicators of renal function. **(E and G)** Representative Masson staining and α -SMA histological immunohistochemistry kidney sections on days 7 and 14. **(F and H)** Quantitative analyses based on images in **(E and G)** (n=3, ** $p < 0.01$, *** $p < 0.001$). **(I–L)** mRNA expression levels of pro-inflammatory cytokines.

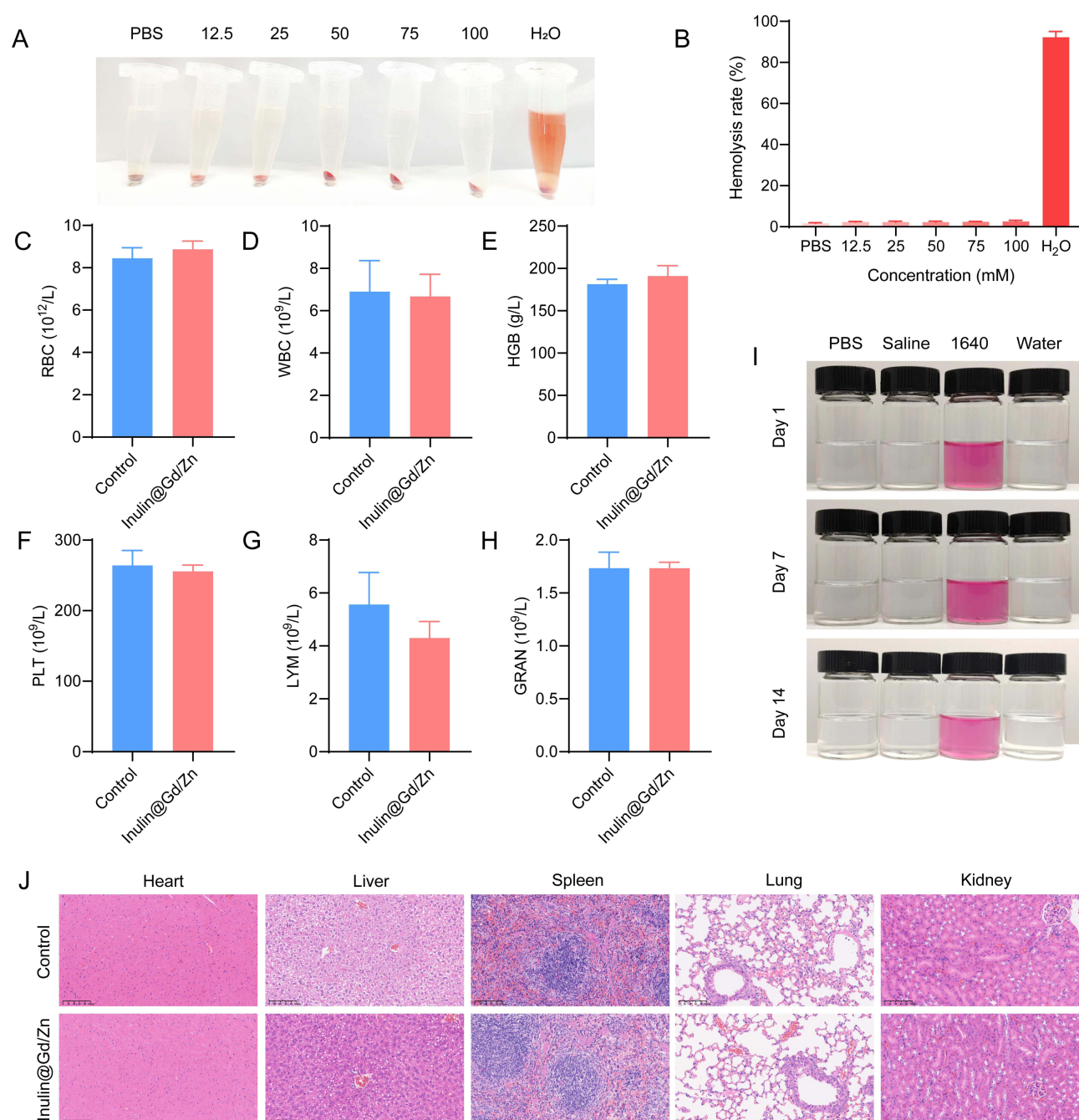


Figure 6 Stability and biocompatibility of Inulin@Gd/Zn NPs. **(A)** Visual inspection of blood samples after exposure to different concentrations of Inulin@Gd/Zn NPs. PBS served as the negative control, whereas H₂O was used as the positive control. **(B)** Percentage of hemolysis after treatment. **(C–H)** Routine blood analysis of mice (n=3) after treatment with PBS or Inulin@Gd/Zn NPs, including red blood cells (RBC), white blood cells (WBC), hemoglobin (HGB), platelets (PLT), lymphocytes (LYM), and granulocytes (GRAN). **(I)** The stability of Inulin@Gd/Zn NPs in PBS, saline, RPMI-1640, and water was evaluated over different time periods. **(J)** H&E staining of major organs.

these conditions (Figure 6I). Furthermore, the histological examination of major organs (including the heart, liver, spleen, lungs, and kidneys) revealed no apparent pathological changes in the treatment group compared to the control group (Figure 6J). Collectively, these findings demonstrated that Inulin@Gd/Zn exhibits a favorable biosafety profile both in vitro and in vivo.

Conclusion

Inulin@Gd/Zn NPs offer a promising novel approach for the clinical application of GBCAs and provide a new direction to address the dual challenges of diagnosis and treatment of patients with CKD. Unlike conventional GBCAs, this nanomaterial features a “theranostic” design—combining therapy and diagnosis—enabling precise renal imaging, while simultaneously mitigating fibrosis progression. This innovative strategy provides a new methodology for early diagnosis of CKD coupled with synchronous treatment. The unique multifunctional synergistic capabilities of these nanoparticles further support their translational potential.

Despite this, its clinical translation still encounters several limitations. First, the core material still poses a risk of gadolinium ion dissociation in an *in vivo* environment, which may lead to potential cytotoxicity and systemic safety concerns. Second, the validation cycle of existing research remains restricted. In this study, we mainly focused on the early intervention of renal fibrosis, with toxicity evaluations concentrated within a short-term observation period of one month. While consistent with the disease stage of the animal model and preliminarily verifying short-term safety, this study did not uncover potential cumulative toxicity or delayed adverse effects over a longer time span. Furthermore, the large-scale controllable fabrication and consistent reproducibility of this nanosystem represent significant remaining challenges. The synthesis process entails multi-step precise regulation, and technical bottlenecks persist in upscaling production capacity, ensuring batch-to-batch consistency, and maintaining high product purity. Moreover, the current synthesis route relies on precious metals and specialized equipment, which leads to high production costs, hindering subsequent preclinical research and development as well as the future market translation of this system.

Abbreviations

GBCAs, gadolinium-based contrast agents; MRI, magnetic resonance imaging; NSF, nephrogenic systemic fibrosis; CKD, chronic kidney disease; UUO, unilateral ureteral obstruction; Gd-DTPA, Gadolinium diethylenetriaminepentaacetic acid; Inulin@Gd/Zn, Inulin@gadolinium/zinc; NPs, nanoparticles; ECM, extracellular matrix; TGF- β 1, tissue growth factor- β 1; TGF- β R1, type 1 tissue growth factor receptor; α -SMA, α -smooth muscle actin; AKI, acute kidney injury; XPS, X-ray photoelectron spectroscopy; FTIR, Fourier transform infrared spectroscopy; PBS, phosphate-buffered saline; CLSM, confocal laser scanning microscopy; DLS, dynamic light scattering; ROS, reactive oxygen species; TEM, transmission electron microscopy; CBC, complete blood count; RBC, red blood cells; WBC, white blood cells; HGB, hemoglobin; PLT, platelets; LYM, lymphocytes; GRAN, Granulocytes; HE, hematoxylin and eosin; FBS, fetal bovine serum; HK-2, human kidney-2; HUVECs, human umbilical vein endothelial cells; DCFH-DA, dichlorodihydrofluorescein diacetate; FITC, fluorescein isothiocyanate; BMDMs, bone marrow-derived macrophages; M-CSF, macrophage colony-stimulating factor; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; RIPA, radio immunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS, tris-buffered saline; ROIs, regions of interest; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; cDNA, complementary DNA; mRNA, messenger RNA; PDI, polydispersity index; SO, sham-operated; GFR, glomerular filtration rate; CREA, creatinine; BUN, blood urinary nitrogen; qPCR, quantitative PCR; IL-6, interleukin 6; IL-1 β , interleukin 1 β ; TNF- α , tumor necrosis factor α ; NF- κ B, nuclear factor-kappa B; CCK-8, cell counting kit-8; ICP-MS, inductively coupled plasma mass spectrometry.

Data Sharing Statement

All data supporting the findings of this study are available within the article and its [Supplementary Information files](#). All relevant data are available from the corresponding author upon request.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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