Antioxidative Hyaluronic Acid–Bilirubin Nanomedicine Targeting Activated Hepatic Stellate Cells for Anti-Hepatic-Fibrosis Therapy

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ABSTRACT: Liver fibrosis is a life-threatening and irreversible disease. The fibrosis process is largely driven by hepatic stellate cells (HSCs), which undergo transdifferentiation from an inactivated state to an activated one during persistent liver damage. This activated state is responsible for collagen deposition in liver tissue and is accompanied by increased intracellular oxidative stress, which contributes to the fibrosis process. To address this problem, we have developed a strategy that combines CD44-targeting of activated HSCs with an antioxidative approach. We developed hyaluronic acid–bilirubin nanoparticles (HABNs), composed of endogenous bilirubin, an antioxidant and anti-inflammatory bile acid, and hyaluronic acid, an endogenous CD44-targeting glycosaminoglycan biopolymer. Our findings demonstrate that intravenously administered HABNs effectively targeted the liver, particularly activated HSCs, in fibrotic mice with choline-deficient L-amino acid-defined high-fat diet (CD-HFD)-induced nonalcoholic steatohepatitis (NASH). HABNs were able to inhibit HSC activation and proliferation and collagen production. Furthermore, in a murine CD-HFD-induced NASH fibrosis model, intravenously administered HABNs showed potent fibrotic modulation activity. Our study suggests that HABNs have the potential to serve as a targeted anti-hepatic-fibrosis therapy by modulating activated HSCs via CD44-targeting and antioxidant strategies. This strategy could also be applied to various ROS-related diseases in which CD44-overexpressing cells play a pivotal role.

KEYWORDS: Anti-liver-fibrosis therapy, Antioxidative therapy, Activated hepatic stellate cells, Bilirubin, Hyaluronic acid, Nanomedicine, Nonalcoholic steatohepatitis, NASH

INTRODUCTION

Liver fibrosis is a severe and life-threatening disease.1 In response to liver damage, hepatic stellate cells (HSCs) are activated at the injury site, initiating a wound-healing process by generating extracellular matrix (ECM) proteins such as collagen and fibronectin.1 However, persistent damage leads to an excessive accumulation of ECM proteins, resulting in liver fibrosis, which is characterized by disrupted liver architecture and impaired functioning.3,4 The excessive deposition of ECM proteins can lead to the development of irreversible cirrhosis. Cirrhosis patients typically experience severe complications including portal hypertension and liver failure with a high likelihood of developing hepatocellular carcinoma in the end stage.5−7 Unfortunately, liver transplantation is the only way to treat end-stage hepatic fibrosis.8

Activated HSCs play a critical role in liver fibrosis by depositing ECM, making them a promising target for antifibrotic therapy.2 Various strategies have been developed to modulate HSCs, including targeting specific molecules overexpressed on their surfaces such as the type VI collagen receptor9,10 and CD44.11−16 Reactive oxygen species (ROS) also have an important role in the liver fibrosis23,24 because ROS are critical in promoting HSC activation,23,24 prolifer-
and migration, and collagen synthesis. Therefore, modulating activated HSCs by addressing oxidative stress is a potentially valuable therapeutic target. However, the paradoxical roles of ROS in liver fibrosis require specific modulation of activated HSCs. While previous studies have sought to address this challenge by developing nanomedicines with HSC- or ROS-modulating activities, they require the use of various experimental drugs as well as complex nanoparticle carriers, thus significantly limiting their successful clinical translation.

To overcome those limitations, we developed hyaluronic acid–bilirubin nanoparticles (HABNs) as a promising antifibrotic nanomedicine that achieves CD44-targeting and antioxidant modulation. HABNs are composed entirely of naturally occurring, biocompatible, and biodegradable materials: bilirubin (BR), an endogenous antioxidant and anti-inflammatory bile acid, and hyaluronic acid (HA), an endogenous glycosaminoglycan biopolymer that targets the CD44 overexpressed on activated HSCs. When they were administered to mice intravenously, the HA shell of the HABNs interacted specifically with CD44, enabling them to target activated HSCs in fibrotic livers but not naive HSCs in healthy livers. Moreover, the BR core conferred potent antioxidant activity, contributing to the beneficial modulation of HSCs. Our results demonstrate that HABNs specifically inhibit the oxidative stress in activated HSCs, leading to the suppression of HSC activity and collagen generation, thereby preventing the progression of liver fibrosis (Figure 1). Overall, our work demonstrates a biocompatible nanomedicine that can simultaneously target...
activated HSCs and control oxidative stress for the treatment of liver fibrosis.

**RESULTS**

Preparation of Hyaluronic Acid–Bilirubin Nanoparticles from a Hyaluronic Acid–Bilirubin Conjugate. To prepare hyaluronic acid–bilirubin nanoparticles (HABNs), we first synthesized a HA-BR conjugate (Supplementary Figure 1). Even though free BR is a potent antioxidant and cytoprotectant that is quite effective against various systemic diseases, its low solubility has hampered the successful clinical translation and use as a therapeutic.\cite{36,38,40} We hypothesized that conjugating BR with very water-soluble HA would dramatically improve its solubility. As expected, although BR was insoluble in water, the HA-BR conjugates were readily dispersed in an aqueous solution (Supplementary Figure 2a).

We also checked how many BR molecules were conjugated with each HA molecule. Our UV/vis analysis revealed an average conjugation density of approximately 4 BR molecules per HA molecule (almost 25 μg/mL of BR in 1 mg/mL of HABNs, Supplementary Figure 2b). HABNs were prepared from the acquired HA-BR conjugate using the film layer method (Supplementary Figure 3a). To investigate whether the HA-BR could self-assemble into a nanostructure, dynamic light scattering (DLS) and transmission electron microscopy (TEM) were used. The DLS analysis showed that the HABNs had a negative zeta-potential (−46.2 ± 4 mV) (Supplementary Figure 3b).

**HABNs Target Activated HSCs.** HCSs, which have important roles in the fibrosis process, express high levels of CD44 on their surfaces when they are activated.\cite{11} To confirm...
that, we first compared CD44 levels among LX-2 cells (human HSCs), HepG2 cells (normal human hepatocytes), and AML12 cells (normal mouse hepatocytes). CD44 levels on LX-2 cells activated by TGF-β treatment were much higher than those in quiescent LX-2 cells (Figure 2a), which expressed minimal CD44 on their surfaces (Figure 2a and Supplementary Figure 4a).

Next, we examined whether the CD44-targeting HABNs could specifically bind to CD44 overexpressed on activated LX-2 cells. To do that, we treated HepG2 cells, AML12 cells, quiescent LX-2 cells, and TGF-β-activated LX-2 cells with HABN−Cy5.5. Higher fluorescence signals from HABN−Cy5.5 were observed in TGF-β-activated LX-2 cells than in HepG2 cells, AML12 cells, and quiescent LX-2 cells (Figure 2b and Supplementary Figure 4). Furthermore, free HA pretreatment dramatically decreased the intracellular uptake of HABNs.
Indeed, compared with Nile red (NR)-loaded HABNs (NR@HABNs), which were readily internalized into TGF-β-activated LX-2 cells, NR-loaded 20K-PEGylated bilirubin nanoparticles (PEG-BNs) showed a much lower degree of internalization (Figure 2c). These results indicate that the HA shell of the HABNs interacted with CD44 overexpressed on activated HSCs, thus allowing the HABNs to target activated HSCs in vitro.

**HABNs Show Antifibrotic Activity against Activated HSCs In Vitro.** Oxidative stress plays an important role in liver fibrogenesis. ROS production is a crucial feature of HSC activation, and ROS can activate HSCs to produce ECM proteins such as collagen.\(^{23-25}\) Interestingly, HABNs exhibited a more robust ROS-scavenging activity compared with both HA and HAOxBNs, the oxidized form of HABNs (Figure 2c). These results indicate that the HA shell of the HABNs interacted with CD44 overexpressed on activated HSCs, thus allowing the HABNs to target activated HSCS in vitro.

To evaluate the antifibrotic activity of HABNs against activated HSCs, we chose a widely used in vitro model for analyzing liver fibrosis, namely TGF-β-activated LX-2 cells. The primary driver of excessive ECM accumulation in patients with liver fibrosis is HSC activation, and one of the key factors responsible for activating HSCs is TGF-β.\(^{2}\) TGF-β-activated LX-2 cells are a widely used model for analyzing liver fibrosis.\(^{41-43}\) We assessed the intracellular ROS production in TGF-β-activated LX-2 cells and the antioxidant activity of HABNs against intracellular ROS. We found that intracellular 2′,7′-dichlorofluorescein fluorescence in TGF-β-activated LX-2 cells was much stronger than the fluorescence signal in quiescent LX-2 cells, indicating that LX-2 cells activated by TGF-β produced more intracellular ROS than quiescent LX-2 cells (Figure 3b). Intracellular ROS levels in a 3D liver fibrosis spheroid model (HSC spheroids) prepared from AML12 cells and LX-2 cells (24:1) were also increased after TGF-β treatment (Supplementary Figure 5a,b). Moreover, we demonstrated that the antioxidant CD44-targeting HABNs significantly reduced ROS production in activated LX-2 cells overexpressing CD44. On the other hand, the CD44-targeting HAOxBNs and antioxidative PEG-BNs (nontargeted BR nanoparticles) had minimal effects on the intracellular ROS levels in activated LX-2 cells (Figure 3b). HABNs also significantly decreased ROS levels in the TGF-β-activated HSC spheroids, and the HAOxBNs and PEG-BNs did not reduce ROS production (Supplementary Figure 5a,b). These results suggest that intracellular uptake mediated by the HA shell and the subsequent antioxidant activity mediated by the BR core are both crucial to the intracellular ROS-scavenging activity of HABNs in activated HSCs.

Because the oxidative stress caused by activated HSCs is highly associated with the enhanced proliferation of HSCs, the viability of LX-2 cells treated with HABNs was evaluated. No significant toxicity was observed in quiescent LX-2 cells treated with HABN at drug concentrations up to 400 μg/mL (Supplementary Figure 6). Notably, however, activated LX-2 cells treated with HABNs exhibited lower viability than that of...
quiescent LX-2 cells (Figure 3c). These results indicate that HABNs can inhibit proliferation of activated LX-2 cells without exhibiting toxicity against quiescent LX-2 cells. The expression of α-SMA in HSCs is highly associated with transdifferentiation to a more activated state, making it a marker of HSC activation. An in vitro confocal analysis revealed that upon activation by TGF-β, both LX-2 cells and HSC spheroids increased their expression of α-SMA (Figure 3d,e and Supplementary Figure 5c). Notably, HABN treatment significantly reduced the expression of α-SMA in activated HSCs, whereas HAoxBNs and PEG-BNs had minimal effects on α-SMA levels in activated HSCs (Figure 3d,e and Supplementary Figure 5c). These results, together with the antioxidant results, suggest that HABN-mediated reduction in oxidative stress is associated with the inhibition of HSC activation.

Collagens I and III are predominantly deposited among various ECM components in fibrotic livers, and TGF-β-activated HSCs play a pivotal role in the generation of collagens I and III. Excessive accumulation of ECM components such as collagen plays a pivotal role in impairing normal liver architecture and functioning and causing liver fibrosis. Because increased collagen expression is also highly associated with enhanced oxidative stress, we investigated the collagen expression levels in both LX-2 cells and HSC spheroids treated with HAoxBNs, PEG-BNs, or HABNs. An
in vitro confocal analysis showed that PEG-BNs and HAoxBNs minimally reduced collagen I expression in activated HSCs, probably due to their limited intracellular uptake (caused by the lack of an HA shell) and their very low antioxidant activity (caused by the oxidized BR core), respectively (Figure 3f and Supplementary Figure 5d). Notably, HABNs significantly inhibited the generation of collagen I in activated HSCs, compared with PEG-BNs and HAoxBNs (Figure 3f and Supplementary Figure 5d).

Taken together, our results show that the antioxidant CD44-targeting HABNs can target activated HSCs and reduce their viability and ECM production.

**HABNs Accumulate in Activated HSCs in Fibrotic Regions of the Liver In Vivo.** A murine nonalcoholic steatohepatitis (NASH) model, in which the mice were fed a choline-deficient l-amino acid-defined high-fat diet (CD-HFD), serves as a dietary NASH model with rapidly progressive liver fibrosis. These CD-HFD-fed mice are considered to be a human-relevant NASH-fibrosis model.41,46

Thus, to determine whether HABNs target fibrotic liver cells, especially activated HSCs, in vivo, we intravenously administered PEG-BN−Cy5.5 or HABN−Cy5.5 to mice fed either a CD-HFD or a normal diet (Figure 4a and Supplementary Figure 7). In mice fed a normal diet, most of the fluorescence signal from HABN−Cy5.5 was localized in the kidney, and minimal signal was observed in the normal liver (Figure 4b,c). In contrast, much higher fluorescence signals from HABN−Cy5.5 were found in the liver of mice kept on a CD-HFD than in their other organs (Figure 4b,c). These results show that HABNs administered intravenously accumulated in the fibrotic liver with minimal accumulation in the normal liver or other major organs (heart, lung, kidney, and spleen). Additionally, the fluorescence signals from HABN−Cy5.5 observed in the livers of mice kept on a CD-HFD were higher than the signals from PEG-BN−Cy5.5 in the livers of mice kept on a CD-HFD. This result indicates the critical role of the HA-shell in targeting the nanoparticles to the fibrotic liver.

Desmin is upregulated in activated HSCs and is widely used as a specific marker of activated HSCs.47−51 To examine whether HABNs accumulated in desmin+ activated HSCs in vivo, we intravenously administered HABN−Cy5.5 to mice fed a CD-HFD or a normal diet. Similar to previous reports,48

![Image](https://doi.org/10.1021/acsnano.3c06107)
desmin expression was upregulated in the livers of mice kept on a CD-HFD, compared to the livers of normal mice (Figure 4d). Notably, no fluorescence signal from HABN−Cy5.5 was detectable in the livers of mice kept on a normal diet, and a strong fluorescence signal from HABN−Cy5.5 overlapping with desmin expression was found in the livers of mice kept on a CD-HFD (Figure 4d). In contrast, the fluorescence signal of PEG-BN−Cy5.5 was scarcely detected within the desmin-expressing regions of the livers of mice fed a CD-HFD (Figure 4e). Taken together, these results suggest that HABNs administrated intravenously colocalize with desmin-expressing activated HSCs in fibrotic regions of the liver, probably due to the activated HSC-targeting ability of the HA shell.

**HABNs Alleviate CD-HFD-Induced NASH Fibrosis.** Based on the excellent HSC-specific antioxidant activity of HABNs in vitro and their targeting of activated HSCs in vivo, we investigated the antioxidant activity of HABNs in vivo. To test this, we fed mice a CD-HFD or a normal diet and then treated them with PBS, PEG-BN, or HABN. The treatment scheme is illustrated in Figure 5a. Western blotting assays and mRNA qRT PCR analyses of fibrotic liver tissue showed that the HABNs significantly decreased α-SMA, ACTA2, and PDGFB expression, indicating HSC inhibition consistent with the in vitro results (Figure 5b–e). In contrast, PEG-BNs did not significantly reduce the markers of activated HSCs. Furthermore, HABNs reduced the burden of deposited ECM, such as TIMP1 and collagens I and III, in the livers of mice kept on a CD-HFD, whereas the PEG-BNs showed minimal activity, suggesting that the HA shell rendered HABNs effective against liver fibrosis, presumably through the HA-mediated targeting of activated HSCs (Figure 5f–h).

Next, we examined whether HABNs could slow the progression of liver fibrosis in a murine CD-HFD-induced NASH fibrosis model. The treatment regimen is illustrated in Figure 6a. Mice fed a CD-HFD and treated with HABNs had significantly lower ALT and AST levels compared to mice fed a CD-HFD and treated with PBS, PEG-BNs, or HABNs. The treatment scheme is illustrated in Figure 5a. Western blotting assays and mRNA qRT PCR analyses of fibrotic liver tissue showed that the HABNs significantly decreased α-SMA, ACTA2, and PDGFB expression, indicating HSC inhibition consistent with the in vitro results (Figure 6b–e). To examine the extent of fibrosis in vivo, immunohistochemistry analyses were performed. The staining results indicate that the livers of mice fed a CD-HFD exhibited signs of NASH fibrosis, such as hepatocellular ballooning, extensive ECM formation, severe centrilobular necrosis, and immune cell infiltration (Figure 6d, f). Furthermore, compared with the control mice fed a CD-HFD, mice treated with PEG-BNs or HABNs had a reduced degree of liver fibrosis. Notably, the ballooning score (a key marker of NASH) showed that the HABNs provided better efficacy than the PEG-BNs (Figure 6d, e). Also, NASH fibrotic mice treated with HABNs exhibited less collagen accumulation and thinner fibrotic septa than NASH fibrotic mice treated with PEG-BNs (Figure 6g). These results suggest that HABN-mediated, activated HSC-specific modulation is very effective in preventing the progression of NASH fibrosis. Lastly, repeated HABN treatments showed no evident indications of toxicity or pathology in vivo (Supplementary Figure 8).

Taken together, our results demonstrate that intravenously administered HABNs exert potent fibrotic modulation activity in a murine CD-HFD-induced NASH fibrosis model.

**DISCUSSION**

In this study, we present our discovery that the administration of antioxidant, CD44-targeting HABNs can specifically inhibit oxidative stress in activated HSCs, leading to the suppression of HSC activity and collagen deposition and consequently effectively preventing the progression of liver fibrosis (Figure 1). As activated HSCs are known to be a key contributor to liver fibrosis, various strategies targeting and modulating their activity have been investigated. Moreover, HSC activation, proliferation, and ECM production are closely linked to increased intracellular oxidative stress, making antioxidants a potential therapeutic option for liver fibrosis. Therefore, our objective was to target oxidative stress induced by activated HSCs with the aim of significantly reducing the progression of liver fibrosis.

Our results demonstrate that intravenously administered HABNs accumulated in activated HSCs in fibrotic liver areas in vivo, possibly due to an interaction between the HA shell of HABNs and CD44, which is overexpressed on activated HSCs (Figure 2, Figure 4, and Supplementary Figure 4). HSCs, a key player in the fibrosis process, exhibit high CD44 levels on their surfaces when activated.13 HABNs exhibited significantly high internalization into activated HSCs with high CD44 expression both in vitro and in vivo, whereas PEG-BNs, which lack CD44 targeting capability, showed minimal localization in activated HSCs. Furthermore, pretreatment with free HA substantially reduced the rate of intracellular uptake of HABNs. These results emphasize the importance of the HA shell for targeting. Notably, HABNs did not accumulate among hepatocytes with low CD44 expression either in vitro or in vivo. These findings collectively support the effective targeting of intravenously administrated HABNs to the liver, particularly to activated HSCs, in mice with CD-HFD-induced NASH fibrosis.

HABNs were taken up by activated HSCs, leading to inhibition of their activation and proliferation as well as collagen production, as evidenced by decreased expression of α-SMA, ACTA2, and PDGFB and reduced production of collagens I and III in LX-2 cells, HSC spheroids, and fibrotic murine livers (Figure 3c–f, Figure 5, and Supplementary Figure 5). This effect was likely due to the strong antioxidant activity of the BR core (Figure 3a, b). Oxidative stress plays a significant role in liver fibrogenesis. ROS are critical in promoting HSC activation, proliferation, migration, and collagen synthesis.23–25 Because the BR core can scavenge ROS, HABNs significantly reduced the level of ROS production in activated HSCs. In contrast, HAOxBNs, which lack the ability to scavenge ROS, and PEG-BNs, which did not target activated HSCs, minimally affected the intracellular ROS levels in activated HSCs. Furthermore, the antioxidative CD44-targeting HABNs decreased the viability of activated LX-2 cells while inhibiting the activation of HSCs. Moreover, the HABNs significantly inhibited the production of collagens I and III in activated HSCs and murine fibrotic livers. In contrast, HAOxBNs and PEG-BNs minimally affected the activation and proliferation of HSCs and did not reduce the level of collagen expression in activated HSCs or murine fibrotic livers. The limited intracellular uptake, caused by a lack of the HA shell, is primarily responsible for the low activity of the non-CD44 targeting PEG-BNs, and the limited activity of the CD44-targeting HAOxBNs due to the oxidation of the BR core by ROS is strongly associated with their loss of antioxidant activity. Therefore, these results suggest that the intracellular uptake mediated by the HA shell and the subsequent antioxidant activity mediated by the BR core are both crucial to the fibrotic activity of HABNs in activated HSCs.
The fibrosis process is largely driven by HSCs, which shift from a quiescent state to an activated state during persistent liver damage. Recent research has demonstrated that homeostatic ROS generation might protect the liver from the progression of liver fibrosis but amplified intracellular oxidative stress in activated HSCs significantly contributes to the fibrosis process. Those paradoxical roles of ROS in liver fibrosis unquestionably plays a crucial role in liver fibrosis. Previous studies have used artificially synthesized materials with complex components and imperative for treating liver fibrosis. Several approaches are available to modulate activated HSCs using antioxidative approaches is highly relevant to the field of liver fibrosis.

**EXPERIMENTAL METHODS**

**Synthesis of the Hyaluronic Acid–Bilirubin (HA-BR) Conjugate.** The HA-BR conjugate was synthesized from an acid form of HA and an amino-ethylene–bilirubin conjugate (AE-BR) using a slightly modified method based on the one previously described by Lee et al.\(^\text{37,38}\) In brief, the acidic form of HA (80 mg/mL), prepared from the sodium salt form of HA (Lifecore Biomedical) using a dialysis method, and N-hydroxysuccinimide (NHS, 40 μmol) were mixed in DMSO (4.8 mL). After 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 140 μmol) was added, the solution was mixed for 10 min at RT. Then, AE-BR (20 μmol), prepared from BR and ethylenediamine using a simple EDC chemistry, was added. Subsequently, the mixture was stirred overnight at RT under nitrogen gas. Finally, the native sodium salt form of HA-BR was obtained through lyophilization. \(^1\)H NMR spectra were acquired on the ANANCE Neo 400 MHz system (Bruker). The BR portion of the HABNs was calculated by using UV/vis spectra from an ELISA reader (Multiskan GO with Cuvette function, Thermo Fisher Scientific).

**Synthesis of PEGylated Bilirubin (PEG-BR).** The PEG-BR conjugate was synthesized using a slightly modified method based on the one previously described by Lee et al.\(^\text{37,38}\) For 10 min at RT, BR (75 μmol) and EDC (33.75 μmol) were mixed in DMSO (0.6 mL) containing triethylamine (225 μL) and NHS (52 μmol). Then, poly(ethylene glycol) 20K-amine (15 μmol, 20K-PEG-NH₂, Nanosoft Polymers) was added, and EDC/NHS chemistry was performed for 4 h under nitrogen gas. After addition of chloroform (50 mL), the organic solution was washed with 0.1 M HCl twice, 0.1 M NaHCO₃ twice, and distilled water twice (50 mL). After the organic layer was evaporated, 50 mL of methanol was added. Unreacted remaining free BR was removed by centrifugation at 3000g for 10 min, and the supernatant was evaporated. Finally, PEG-BR was obtained through dialysis, following the procedure outlined in the HA-BR conjugate section and then lyophilization.

**Synthesis of the HA-BR–Cy5.5 Conjugate.** Cy5.5 conjugated HA-BR was synthesized using a slightly modified method based on the one previously described by Lee et al.\(^\text{37,38}\) In brief, the acid form of HA-BR (10.5 μmol), prepared from the native sodium salt form of HA-BR through dialysis against 0.01 M HCl and water, was dissolved in DMSO (0.8 mL) overnight. NHS (2 μmol) and EDC (2 μmol) were then added to the mixture and stirred for 10 min at RT. Cy5.5-NH₂ (0.1 μmol, AAT Bioquest) was then added and stirred overnight, followed by dialysis following the procedure outlined in the HA-BR conjugate section. The resulting solution underwent lyophilization, yielding the HA-BR–Cy5.5 conjugate.

**Preparation of HABNs, PEG-BNs, and Cy5.5-tagged HABNs.** HABNs, PEG-BNs, and Cy5.5-tagged HABNs were synthesized following a method previously described by Lee et al.\(^\text{37,38}\) HA-BR, PEG-BR, or HA-BR–Cy5.5 were dissolved in water or PBS and then sonicated for 10 min at RT. Finally, HABNs, PEG-BNs, or Cy5.5-conjugated HABNs were obtained after filtration through a 0.45 μm filter membrane. The hydrodynamic size, zeta potential, and TEM morphology of HABNs, PEG-BNs, and Cy5.5-conjugated HABNs were examined using a Zetasizer Nano ZS90 (Malvern Instruments Ltd.) and a JEM-2100F (JEOL USA). Prepared HABNs, PEG-BNs, or Cy5.5-conjugated HABNs were diluted in PBS or a culture medium for further studies.

**Cell Culture.** The HepG2 human hepatocyte, AML12 murine hepatocyte, and LX-2 human hepatic stellate cell lines were obtained from ATCC. All cell lines tested negative for mycoplasma contamination. AML12 cells were cultured in DMEM/F12 (Welgene) containing 10% (v/v) fetal bovine serum (Corning), 5 mg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone; HepG2 cells were cultured in RPMI medium (Welgene); and LX-2 cells were cultured in DMEM (Welgene) containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and 100 IU/mL penicillin/streptomycin (Gibco) were cultured in a humidified 5% CO₂ atmosphere at 37 °C.

**Preparation of the Native Polymer form of HAoxBR.** To acquire HAoxBR, HABNs were oxidized with 2,2’-azobisis(2-methylpropionamide) dihydrochloride (AAPH, 100 μM) in water over 1 day at 37 °C. Then, dialysis was carried out against distilled water for 24 h to eliminate any remaining AAPH. Subsequently, the solution underwent lyophilization, yielding HAoxBR.

**Preparation of NR@HABNs and NR@PEG-BNs.** First, 0.5 mg of Nile red (NR, Tokyo Chemical Industry Co., Ltd.) was added to 1 mg of HABNs in 1 mL of chloroform, and then sonication was performed for 10 min at RT. After the organic solvent was evaporated, filtration was carried out through a filter membrane (0.45 μm) to eliminate any unloading NR, resulting in the acquisition of evenly sized NR-loaded HABNs. The amount of NR loaded onto the nanoparticles was quantified using HPLC (Agilent 1260, Agilent Technologies).

**Intracellular ROS.** LX-2 cells were seeded in 96-well plates (4.0 × 10⁴/well). After 1 day of incubation at 37 °C, the cells were treated with HABNs (0.5 mg/mL), HAoxBRs (0.5 mg/mL), PEG-BR (0.5 mg/mL), or fresh medium for 24 h. TGF-β1 (2 ng/mL) was added to the cells 9 h before the end of the nanoparticle treatments. The cells were then incubated for 1 h with diaceteyldichlorofluorescein (DCFDA, 30 μM) in fresh medium. After washing the cells with PBS, we observed the fluorescence signals generated from DCFDA by using a fluorescence microscope.
oxidation using a microplate reader (Twinfinite 200 PRO, Tecan) at 488 nm excitation and 525 nm emission.

**In Vitro Confocal Microscopy.** For the CD44 expression and nanoparticle uptake assay, HepG2, AML12, and LX-2 cells were seeded on separate coverslips in 24 well plates (1.0×10^5/well). After a 2 day incubation at 37 °C, LX-2 cells were incubated with TGF-β1 (2 ng/mL, Bio-Techne) or control medium for 24 h. The cells were then treated with HABN–Cy5.5 (30 μg/mL), free NR (1 μg/mL), NR@HABNs (NR, 1 μg/mL; HABNs, 62.5 μg/mL), NR@PEG-BNs, and FITC-conjugated anti-CD44 antibody (1:100 dilution; Clone IM7, no. 103016, BioLegend), or control medium for 1 h. Subsequently, a fixation step with 4% paraformaldehyde was performed for 5 min. After being counterstained with Hoechst 33342 for 10 min and examined using a Nikon A1R confocal microscope. In the particle uptake competition assay, the cells were pretreated with 5 μg/mL free HA overnight before the nanoparticle treatment.

For the α-SMA and collagen-1 expression assay, LX-2 cells were treated with HABNs (0.2 mg/mL), HAoxBNs (0.2 mg/mL), PEG-BNs (0.2 mg/mL), or fresh medium for 24 h. TGF-β1 (2 ng/mL) was treated with the LX-2 cells 9 h before the end of the nanoparticle treatment. After the medium was replaced with fresh medium, LX-2 cells were further incubated overnight at 37 °C and then fixed with ice-cold 100% methanol for 5 min. After fixation, the cells were treated with PBS containing 0.25% Triton X-100 for 10 min and then treated with a blocking buffer [1% BSA and glycine (22.52 mg/mL) in PBST (PBS + 0.1% Tween 20)] for 0.5 h. The cells were then incubated with anti-α-SMA rabbit antibody (1:100 dilution; no. ab5694, Abcam) or collagen-1 rabbit antibody (1:1000 dilution; no. ab34710, Abcam) in 1% BSA in PBST for 2 h. After being washed again with PBST, the cells were incubated in the dark with secondary anti-rabbit goat antibody (1:200 dilution; no. ab150077, Abcam) in 1% BSA in PBST in a humidified chamber for 1 h at 37 °C. The cells were then counterstained with Hoechst 33342 for 5 min. Finally, samples were examined by using a Nikon A1R confocal microscope.

**In Vitro 3D Fibrosis Model Formation.** A 3D liver fibrosis model procedure was described using previously by Pingitore et al. 53 To form the 3D spheroids, AML12 and LX-2 cells (24:1 ratio) were seeded at 4.0×10^5 cells and cocultured in a 96-well round-bottom plate (no. 34896, SPL) with high-glucose DMEM (no. LM 001-05, Welgene) containing 10% (v/v) FBS (no. 35-015-CV, Corning). After spheroid formation was allowed for 8 days after seeding, the AML12/LX-2 spheroids were exposed to TGF-β1 (10 ng/mL) to mimic the pathophysiology of hepatic fibrosis.

**In Vitro 3D Fibrosis Intraacellular ROS.** 3D spheroids that had been cultured for 8 days were treated with HABNs (10 μM), HAoxBNs (10 μM), or PEG-BNs (10 μM) for 48 h with or without TGF-β1 (10 ng/mL). On day 10, all spheroids were transferred into Nunc F96 MicroWell black polystyrene plates (no. 237105, Thermo Scientific) and incubated for 1 h with 20 μM dichlorohydrofluorescein diacetate (DCFHDA) in fresh phenol-red-free medium. After washing the spheroids with PBS, we measured the fluorescence signals generated from DCFHDA by oxidation using a microplate reader (Infinite 200 PRO, Tecan) with an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

**In Vitro 3D Spheroid Immunofluorescence Assay.** To form 3D spheroids, AML12 and LX-2 cells (in a 2:4:1 ratio) were seeded at 4.0×10^5 cells and cultured in a 96-well round-bottom plate for 8 days. On day 8, the 3D spheroids were treated with HABNs (10 μM), HAoxBNs (10 μM), or PEG-BNs (10 μM) for 48 h in the absence or presence of TGF-β1 (10 ng/mL). The 3D spheroids were fixed in 4% paraformaldehyde solution for 1 h and subsequently incubated overnight in a 20% sucrose solution. Then, all spheroids were embedded in the OCT compound and prepared as frozen sections (8 μm thick). After being washed with 1× ice-cold PBS, the sections were blocked using a blocking solution (1% BSA, 300 mM glycine, PBST) and incubated with α-SMA antibody (1:1000 dilution; no. ab5694, Abcam) or collagen-1 antibody (1:1000 dilution; no. ab34710, Abcam) in PBST for 2 h. After being washed again with 1× PBS, the spheroids were treated with Alexa Fluor 488-conjugated secondary antibody (1:100, no. 4412, Cell Signaling Technology) for 1 h. After being washed with PBS, the spheroids were counterstained with DAPI (0.1 μg/mL in PBS) for 10 min. Finally, the samples were monitored using a fluorescence microscope (Aпотоме, Carl Zeiss Co. Ltd.) and analyzed with Zen Pro software.

**CCK-8 Assay.** LX-2 or AML12 cells were seeded onto 96-well plates (1×10^5/well). After 1 day of incubation at 37 °C, the cells were treated with 100 μL of HABNs (50, 100, 200, 400 μg/mL) or fresh medium for 15 h. TGF-β1 (2 ng/mL) was treated with the LX-2 cells or AML12 cells 9 h before the end of the nanoparticle treatment. After the removal of the medium, the cells were treated with a culture medium supplemented with CCK-8 (10:1 ratio, Abbkine) for 1–2 h at 37 °C. Finally, an ELISA reader (Multiskan GO with Cuvette function, Thermo Fisher Scientific) was used to measure the absorbance at 450 nm.

**Animals.** All work performed on animals was in accordance with and approved by the Institutional Animal Care & Use Committee (IACUC) at Ewha Womans University (IACUC No. 19-041). All animals were obtained from Raon-bio (Korea) as mixed littermates and housed under pathogen-free conditions in the animal facility at the College of Pharmacy, Ewha Womans University.

**Preparation of Cy5.5-Loaded PE Gyelated Bilirubin Nanoparticles (Cy5.5@PEG-BN).** First, Cy5.5-COOH (0.5 mg, HYD1040, MedChemExpress) was added to PEG-BNs (1 mg/mL) in chloroform, and then sonication was performed for 10 min at RT. After evaporation of the organic solvent, we performed dialysis overnight against distilled water to remove the remaining unloaded Cy5.5. The amount of Cy5.5 loaded onto the nanoparticles was measured using UV/vis spectra from an ELISA reader (Multiskan GO with a Cuvette function, Thermo Fisher Scientific).

**In Vivo Imaging System (IVIS) Imaging.** To investigate the distribution of the HABNs and PEG-BNs in the liver, C57BL/6J mice (male, 6-week-old) kept on a normal diet (standard mouse pellet) or CD-HFD (a-amino acid diet containing 60% of calories from fat, with 0.1% methionine and devoid of additional choline, no. A0690, Research Diet) for 6 weeks were iv-injected with 3.5 mg/kg of HABN–Cy5.5 (with corresponding amount of 4.3 μg of free Cy5.5 in 1 mg of HABN–Cy5.5) or 3 mg/kg of Cy5.5-loaded PEG-BN (with a mass of free Cy5.5 equiv to that in the HABN–Cy5.5) on day 42. Six hours after treatment, all mice were sacrificed, and major organs were excised immediately. The fluorescence intensities of the major organs were monitored using an in vivo imaging system (IVIS, PerkinElmer) under a Cy5.5 filter channel.

**In Vivo Immunofluorescence Assay.** Liver tissues were freshly excised from the mice, fixed in 4% paraformaldehyde, and then prepared as frozen tissue sections. The sections were incubated with PBS containing 0.1–0.25% Triton X-100 (Sigma-Aldrich) for 10 min. The sections were then blocked with a blocking solution (1% BSA, 300 mM glycine, PBST) and incubated with antibody (desmin–FITC, no. NBP2-54503F, Novus Biologicals) in PBST containing 1% BSA overnight at 4 °C. Following another wash with 1× PBS, DAPI solution (0.1 μg/mL) was added for 10 min. The samples were monitored using a fluorescence microscope (Aпотоме, Carl Zeiss Co., Ltd.) and analyzed with Zen pro software.

**In Vivo Hepatic Fibrosis Model Study.** C57BL/6J (male, 6-week-old) mice kept on a normal diet or a CD-HFD for 6 weeks were administered 12 mg/kg (iv) HABNs, PEG-BNs, or PBS every 3 days for a total of 7 times. On day 42, blood was collected for the blood parameter analysis, and the murine livers were excised. Liver weight and size were assessed, and the liver tissues were used for further in vivo tissue analyses.

**In Vivo Western Blot Analyses.** Liver tissues were lysed in lysis buffer (20 mM HEPES at pH 7.0, 0.15 M NaCl, 10% glycerol, 0.5% NP40, 1% phenylmethylsulfonyl fluoride (PMSF), 1% cocktail inhibitor (GenDEPOT), 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, and 5 mM sodium fluoride). The Western blot analysis procedures followed the general protocols described elsewhere. Protein bands were visualized using a LAS-3000 instrument (FUJIFILM Corporation) with ECL solution reagent (GE Scientific).
Healthcare. Images were captured and analyzed with Multi-Gauge V3.0 software. Anti-α-SMA antibody (no. 19245) and anti-vinculin antibody (no. PM088) were from Cell Signaling Technology and MBL, respectively.

**In Vivo Immunohistochemistry Assay.** Liver tissues freshly excised from mice were immediately fixed in 4% paraformaldehyde and used to make paraffin-embedded block sections for immunohistochemistry (IHC). IHC procedures followed the general protocols described elsewhere. Sections were stained with hematoxylin (no. HMM999, Scy Tek, Inc.) and eosin (no. 318906, Sigma-Aldrich) or Sirius red (Picro Sirius red stain kit; no. ab150681, Abcam) according to the manufacturers’ instructions. All images were captured using a microscope (Carl Zeiss Co. Ltd., Jena, Germany) at 200x magnification at the Ewha Drug Development Research Core Center and analyzed with ImageJ software. IHC staining was evaluated quantitatively using the IHC score. For this staining, α-SMA antibody (no. 19245, Cell Signaling Technology) was diluted according to the manufacturer’s recommendations.

**Quantitative Real-Time PCR (qRT PCR) Assay.** Total RNA was extracted from liver tissues using Tri-RNA reagent (FAVORGEN manufacturer’s recommendations. PrimeScript RT reagent kit (no. RR037A, Takara Bio Inc.) following the manufacturers’ instructions. All images were captured using a microscope (Carl Zeiss Co. Ltd., Jena, Germany) at 200x magnification at the Ewha Drug Development Research Core Center and analyzed with ImageJ software. IHC staining was evaluated quantitatively using the IHC score. For this staining, α-SMA antibody (no. 19245, Cell Signaling Technology) was diluted according to the manufacturer’s recommendations.

**Statistical Analysis.** All experiments were conducted at least twice with duplicate repeated measures. The results are presented as mean ± SEM. A one-way ANOVA followed by Tukey’s HSD multiple comparison post hoc test was used to assess differences among groups. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA) was used for the statistical analyses. Experiments were independently replicated several times as specified in each figure legend.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nano.3c06107.

Synthesis of hyaluronic acid-bilirubin conjugate (HABR); characterization of HA-BR; preparation and characterization of hyaluronic acid–bilirubin nano-particles (HABNs); HABN targeting of TGF-β-activated LX-2 cells; HABN antifibrotic activity in the in vitro 3D fibrosis model consisting of hepatocytes and HSCs; HABN lack of toxicity in quiescent LX-2 and AML12 cells; schematic illustration of the synthesis of HABN–Cy5.5; safety profile of HABN therapy (PDF)

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**Author Contributions**

J.S. and S.P. contributed equally to this work. J.S., S.P., Y.K., and Y.L. designed the experiments. J.S. and S.P. performed all in vivo experiments. Y.K. and Y.L. performed all in vitro experiments. J.S. and S.P. performed the statistical analyses. All authors contributed equally to the writing of the manuscript.

**Notes**

The authors declare no competing financial interest.

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