Magnetically-Targeted Nanoparticle-Guided M2 Macrophage Polarization For Fracture Healing Promotion

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Research

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Abstract

Background: Macrophages are essential for fracture healing, acting mainly through remodeling of the extracellular matrix and promotion of angiogenesis. The role of macrophages in regulating osteogenic differentiation, particularly that of the M2 phenotype, is increasingly researched. Baicalein (BCL) had also been shown to have pro-fracture-healing effects.

Results: In this study, we developed mesoporous silica and Fe_3O_4 composite-targeted nanoparticles loaded with BCL (BCL@MMSNPs-SS-CD-NW), that could be magnetically delivered to the fracture site. These induced macrophage recruitment in a targeted manner, polarizing them towards the M2 phenotype, and thereby inducing MSCs towards osteoblastic differentiation. The mesoporous silicon nanoparticles (MSNs) were prepared with surface sulfhydrylation and amination modification, and the mesoporous channels were blocked with β -cyclodextrin. The outer layer of the mesoporous silicon was added with an amantane-modified NW targeting peptide to obtain the targeted nano-system. After entering macrophages, BCL could be released from nanoparticles since the disulfide linker could be cleaved by intracellular glutathione (GSH) resulting in the removing of CD gatekeeper, which is a key element in the pro-bone-remodeling functions, such as anti-inflammation and induction of M2 macrophage polarization to facilitate osteogenic differentiation.

Conclusions: This nano-system passively accumulated in the fracture site, promoting osteogenic differentiation activities, highlighting a potent therapeutic benefit with high biosafety.

Background

Although fracture healing occurs in most cases without delay, 5–10% of patients suffer from delayed healing or non-union, severely compromising the patients' quality of life, exerting a societal burden and becoming a severe global public health challenge [1]. Despite the development of advanced therapeutic strategies for reversing non-union, the benefits are not as extensive as required [2, 3]. Macrophages are crucial cells in the inflammatory cascade and an essential component in the regeneration of various tissues and organs including bone [4, 5]. For example, it was demonstrated that macrophage scavenger receptor 1 is capable of promoting fracture healing through promotion of the recruitment and osteogenic differentiation of mesenchymal stem cells (MSCs) [6]. A better understanding of the cross-talk between macrophages and MSCs would revolutionize the development of fracture healing therapy.

Baicalein (5,6,7-trihydroxyflavone, BCL)—an active ingredient extracted from the traditional Chinese herb *Scutellaria baicalensis* *Georgi*—has been shown to have anti-inflammatory, anti-oxidant, anti-cancer and anti-diabetic functions and is widely employed in a variety of conditions [7–9]. In bone fracture healing, BCL has been reported to induce osteoblastic differentiation, and suppress osteoclast maturation regulating bone metabolism [10]. Furthermore, the positive effect of BCL on M2 macrophage polarization has also been reported [11]. In a recent study, Yang et al reported that BCL was able to ameliorate neuroinflammation and neuronal damage through promotion of M2 macrophage polarization [12].

However, BCL has rarely been used in the treatment of bone fractures largely due to limitations in its targeted delivery to macrophages in the fracture site, as well as its toxic effect on other organs.

Research has highlighted the promising use of nanomedicine in osteogenic differentiation, with recent years seeing a surge in the development of nanomedicines for bone repair, in an effort to overcome the weaknesses of currently available strategies [13–15]. A number of nano-drug delivery systems have been designed to effectively locally deliver various drugs into fracture sites, with improved drug concentration and decreased side-effects [16–18]. Moreover, targeted drug delivery systems have been designed to enhance the therapeutic effects via surface modifications of specific recognition ligands [19, 20]. Despite extensive strides, the field is still in its infancy, exemplified by a scarcity of effective nanomedicines for bone repair.

In the present study, a multifunctional nanocarrier based on magnetic mesoporous silica nanoparticles (MMSNPs) loaded with BCL (BCL@MMSNPs-SS-CD-NW) was designed for fracture healing. As illustrated in Scheme 1A, the BCL was loaded into MMSNPs via a series of decoration such as sulfhydrylation, alkynylation, and the cyclodextrin is modified as the gatekeeper for BCL. Furthermore, the peptide NW, a peptide that could target to macrophages, is modified on the surface of MMSNPs via the interaction of host-guest effect between adamantane and cyclodextrin. In this nanoplatform, BCL@MMSNPs-SS-CD-NW could accumulate in the fracture area after intravenous injection due to the external magnetic field applied at fracture site. The NW peptide-mediate macrophage targeting then promotes the uptake of BCL@MMSNPs-SS-CD-NW into macrophages. Subsequently, the BCL is released from MMSNPs due to the removing of CD resulting from the cleavage of disulfide linker by intracellular GSH. Meanwhile, the content of intracellular ROS will be decreased and the NF- κ B pathway will be inhibited (Scheme 1B), which will lead to the polarization of M2 phenotype macrophages from BMDMs and promote the differentiation of MSCs (Scheme 1C). To gain further understanding, the pro-healing mechanisms of BCL@MMSNPs-SS-CD-NW were also studied. Finally, the therapeutic effects and safety of BCL@MMSNPs-SS-CD-NW were assessed following systemic administration in an *in vivo* fracture mouse model. This work demonstrated for the first time that BCL@MMSNPs-SS-CD-NW can potentially be used in the treatment of bone fractures.

Results

Preparation and characterization of BCL@MMSNPs-SS-CD-NW

The multifunctional nanocarrier MMSNPs-SS-CD-NW was synthesized according to literatures with slight modifications [21,22] and the detail procedure was described in *Materials and Methods*. The images of transmission electron microscopy (TEM) showed uniform and well-dispersed MMSNPs with a highly ordered lattice array (Figure 1A). The diameter exhibited about 100 nm, which was suitable for long time circulation. The dynamic light scattering (DLS) was then detected. Similar to the result of TEM, the DLS shows positive dispersity and that the hydrodynamic size of the MMSNPs is 217.7 ± 4.23 nm (Figure 1B). The Zeta potentials of intermediate products during the preparation of BCL@MMSNPs-SS-CD-NW

changed obviously (Figure 1C), which indicates the successful decoration of nanoparticles at every step. Moreover, The surface functionalization of intermediate products was analyzed by fourier transform infrared spectroscopy (FT-IR). As shown in Figure S1A (Supporting information), the appearance of absorption of the CTAB templates at $2800\text{--}3000\text{ cm}^{-1}$ and it disappears in Figure S1B (Supporting information), verifying the CTAB templates was successfully extracted. Besides, the typical alkyne moieties absorption peak at 2125 cm^{-1} was displayed in Figure S1E (Supporting information), which indicated the MMSNPs-SS-alkyne was synthesized. We also monitor the diameter and zeta potential of MMSNPs-SS-CD-NW nanoparticles in H₂O, DMEM and PBS with 10% fetal bovine serum. The diameter and zeta potential was almost unchanged within 48 hours (Figure S4 and Figure S5). These results confirmed the good stability of PpIX-1MT nanoparticles in physiological conditions. The results of BCL released from BCL@MMSNPs-SS-CD-NW *in vitro* is shown in Figure 1D. The concentration of BCL was determined by ultraviolet–visible (UV-vis) absorption spectroscopy at a wavelength of 325 nm. The release of BCL was almost negligible for 120 minutes in the absence of GSH, indicating that BCL could be efficiently stuck in the pore of MMSNPs by CD for a long time. On the contrary, the BCL was released rapidly from MMSNPs due to the removal of CD via a cleavage of disulfide bonds in the presence of GSH. Furthermore, the release of BCL was accelerated with increasing GSH concentration. About 20% of BCL was released rapidly within 6 minutes in the presence of 1mM GSH while about 60% of BCL was detected in the presence of 10mM GSH. Additionally, the magnetic targeting function of MMSNPs *in vitro* was investigated. As shown in Figure 1E, under the stimulation of applied magnetic field, MMSNPs reacted rapidly. Only 10 minutes post-stimulation of magnetic field, almost all the MMSNPs were accumulated on one side of the magnetic field. In addition, T2-weighted Magnetic Resonance Imaging (MRI) of MMSNPs-SS-CD-NW on a 7.0 T MR spectrometer was investigated. As shown in Figure 1F, the T2 signal intensity revealed a concentration-dependent darkening effect and decreased significantly with increasing Fe³⁺ concentration, which demonstrates the feasibility that our designed MMSNPs could be as a contrast agent for MR imaging.

BCL induces M2 macrophage polarization via inhibition of the ROS/NF- κ B signaling

To elucidate the underlying mechanism of BCL's effect on macrophage polarization, we measured the expression of phenotypic markers associated with bone marrow-derived macrophages (BMDMs) using flow cytometry (**Figure S2**), and investigated how different concentrations of BCL affect M2 macrophage polarization *in vitro*. A cellular oxidative microenvironment was induced with 100 μM H₂O₂, and BMDMs were prepared with phosphate buffered saline (PBS), and 5, 10, 20, or 40 $\mu\text{g}/\text{mL}$ BCL, respectively. Phenotypic markers of M1 and M2 macrophages including iNOS and Arg-1 were evaluated Western Blot analysis 48 hours post-treatment. Compared with the PBS group, significantly higher level of Arg-1 and lower level of iNOS were detectable in the BCL-treated groups, and the most prominent effect was noted in the 40 $\mu\text{g}/\text{mL}$ BCL-treated group (**Figure 2A-B**). Furthermore, we sought to elucidate the underlying molecular mechanism of BCL's regulation via detection of ROS and NF- κ B related genes. As seen in **Figure 2C**, the ROS fluorescence was gradually decreased after the BCL treatment, with the statistical analysis suggesting a consistent result (**Figure 2D**). Similarly, we collected

BMDMs from BCL-treated groups with different concentrations to study NIK activation, p65 phosphorylation and I κ B kinase degradation in the cells by Western Blot. As shown in **Figure 2E**, compared with BMDMs treated with PBS, the expression of NIK in BMDMs decreased significantly in correlation with the increased concentration of BCL treatment for 24 hours. In the same way, the ratio of phosphorylated p65 (pi-p65)/p65 and phosphorylated I κ B (pi-I κ B)/I κ B also declined statistically (**Figure 2F**). Increased BCL concentrations induce degradation of NIK, hence, BCL treatment suppressed the activation of the ROS/NF- κ B signaling pathway in BMDMs and inhibited the production of pro-inflammation cytokines, such as TNF- α and IL-1 β , in agreement with prior research [23].

Nuclear factor-activated κ -light chain enhancement of B cells (NF- κ B) is a protein complex that controls DNA transcription, cytokine production, and cell survival [24]. NF- κ B is found in almost all cell types and is involved in the cellular stress response, and it was well documented that activation of ROS/NF- κ B signaling is closely related with inflammation [25-27]. Prior studies have reported the robust anti-inflammatory and ROS scavenging effect of BCL, and M2 macrophage is one of the most important anti-inflammatory cell types [28,29]. Thus, in the present study, we tested how BCL affects the expression of NF- κ B and the release of ROS, as well as the macrophage subtype polarization. Consequently, our results suggested a marked suppressed effect of BCL on ROS/NF- κ B signaling, and with the stimulation of BCL, BMDMs was effectively induced to M2 macrophage polarization.

M2 macrophage polarization induces MSCs towards osteoblastic differentiation *in vitro*

We next investigated how M2 macrophage polarization affects osteogenic differentiation *in vitro*. A cell co-culture model system was used, wherein M1 or M2 macrophages are placed in the upper chamber of a transwell chamber system. MSCs were added to the lower chamber, and we assessed the osteogenic-related markers in the lower chamber after co-culturing for 48 hours (**Figure 3A**). As shown in **Figure 3B-C**, the osteogenic-related markers, including Collagen I, osteocalcin (OCN), and runt-related transcription factor 2 (Runx2), were significantly enriched in the M2 macrophage co-cultured group compared with the PBS-treated and M1 macrophage co-cultured group. In addition, to study the impact of M2 macrophage polarization on extracellular matrix mineralization, MSCs in the lower chamber were continuously cultured for 7 or 21 days and then assessed with ALP or alizarin red staining, revealing enhanced mineral deposition in M2 macrophage co-cultured-cells, and reduced mineral deposition in M1 macrophage co-cultured-cells (**Figure 3D-G**).

Several mechanisms are known to contribute to osteoblastic differentiation [30]. Prior studies suggested that M2 macrophage polarization may contribute to osteoblastic differentiation and bone mineralization [31,32]. Macrophages have two sub-phenotypes, and the polarization state of macrophages is dependent on factors in the microenvironment including several cytokines and chemokines [33]. Mechanistically, M2 macrophages secrete osteogenic factors that stimulate the differentiation and activation of MSCs, and subsequently increase bone mineralization. Recent data have demonstrated that M2 macrophages polarization are capable to stimulate MSCs into mature osteoblasts and increase bone mineralization *in*

vitro [34,35]. Consistently, our study indicated that interventions that favor M2 macrophage polarization may be a novel approach for bone fracture treatment.

BCL@MMSNPs-SS-CD-NW has good biocompatibility

Cell viability is one of the crucial factors when assessing bioactive nanoparticles as it directly influences the final cellular activities, including proliferation, apoptosis, and differentiation. We performed a cell counting kit-8 (CCK-8) assay to evaluate the viability of the cultured BMDMs, finding no obvious growth difference among the experimental groups 1, 3, 5 and 7 days after incubation (**Figure 4A**). BMDMs proliferated well in the different groups, with or without nanoparticles, suggesting that the applied nanoparticles including BCL@MMSNPs-SS-CD-NW were not harmful to BMDMs. Furthermore, the apoptosis rate of BMDMs in different groups was assessed using flow cytometry, and our results indicated that the apoptosis rate of BMDMs was not markedly increased after adding nanoparticles in the medium compared with the control group (**Figure 4B-C**). Moreover, cell status was checked using live/dead cell staining five days after culture. All the cells were morphologically similar, either in the presence of cell culture medium or nanoparticles (**Figure 4D-E**). Together, the prepared nanoparticles displayed good cytocompatibility, and did not affect the proliferation BMDMs.

Targeted delivery of BCL@MMSNPs-SS-CD-NW to BMDMs in the fracture site

As a noninvasive diagnostic technique with adequate resolution, small animal MRI examination has been recognized as a feasible tool for early detection of nanoparticles *in vivo*. To demonstrate the magnetic targeting of BCL@MMSNPs-SS-CD-NW to the fracture site, fracture sites in right legs were exposed to an external magnetic field, and the fracture sites in left legs were treated as the control group without external magnetic power (**Figure 5A**). The Inductively Coupled Plasma Mass Spectrometry (ICP-MS) result suggested that the nanoparticles was markedly attracted to the fracture sites in right legs on 12 hours after the magnetic guidance reflected by the Fe^{3+} content in the fracture sites (**Figure 5B**). Similarly, T2 signal intensity suggested that the darkening effect of the fracture sites in right legs was significantly prominent compared with the fracture sites in left legs (**Figure 5C-D**). Clearly, the magnetic targeting could enhance the efficacious accumulation of BCL@MMSNPs-SS-CD-NW in the fracture site, which is important for fracture-targeted therapy. Next, we evaluated the *in vivo* biocompatibility of BCL@MMSNPs-SS-CD-NW via intravenous injection of PBS, BCL, or BCL@MMSNPs-SS-CD-NW in healthy mice twice a week for 6 weeks. We found no apparent histomorphometric changes in the heart, liver, lung, kidney, or spleen in the BCL@MMSNPs-SS-CD-NW group, while apparent histomorphometric injury of liver and lung was noted in the free BCL group. This suggests that compared with free BCL intravenous-injection, BCL@MMSNPs-SS-CD-NW is well-tolerated, with little to no acute, systemic toxicity (**Figure 5E**). In all, our results suggest the effective targeting function of BCL@MMSNPs-SS-CD-NW to BMDMs with high biosafety.

BCL@MMSNPs-SS-CD-NW induces M2 macrophage polarization

Furthermore, to assess the targeting ability of the NW polypeptide, BMDMs were pre-treated with NW polypeptide in a different concentration (0, 10 $\mu\text{mol/L}$, 25 $\mu\text{mol/L}$, and 50 $\mu\text{mol/L}$) for 60 minutes. Subsequently, BCL@MMSNPs-SS-CD-NW was labeled with FITC fluorescence (green) and added into the BMDMs in different groups, and flow cytometry (FCM) was used to achieve the quantitative results of fluorescence intensity of the groups. As shown in **Figure 6A-B**, the highest surge of fluorescence intensity in non-blocking group was appeared at 1 hour after the addition of FITC-labeled BCL@MMSNPs-SS-CD-NW. Thus, we selected the 1 hour as the time-point to detect the fluorescence intensity among the different groups by FCM, indicating that fluorescence intensity in the non-blocking group was significantly enhanced compared with the NW peptide blocking groups, and 50 $\mu\text{mol/L}$ NW-pre-treated groups suggested the best blocking efficiency (**Figure 6C-D**). Consistently, a similar result was observed in the confocal microscopy examination, suggesting more efficient uptake of labeled BCL@MMSNPs-SS-CD-NW in non-blocking group compared to the NW-blocking groups (**Figure 6E**). All these results indicated a sufficient targeting rate of NW polypeptide to macrophages, which is an important basis for investigating the molecular mechanisms of BCL@MMSNPs-SS-CD-NW.

We next investigated the ability of BCL@MMSNPs-SS-CD-NW to directly affect M2 macrophage polarization by producing a cellular oxidative microenvironment using 100 μM H_2O_2 , and BMDMs were treated with PBS, BCL@MMSNPs-SS-CD-NW, and free BCL (40 $\mu\text{g/mL}$), respectively. Phenotypic markers of M1 and M2 macrophages including iNOS and Arg-1 were evaluated by cytometry analysis and Western Blot analysis 48 hours post-treatment. Compared with the PBS group, significantly higher level of Arg-1 and lower level of iNOS were detectable in the BCL@MMSNPs-SS-CD-NW and free BCL groups (**Figure 6F-I**). Together, our results indicated that BCL@MMSNPs-SS-CD-NW is capable of promoting M2 phenotype polarization via targeted delivery and release of BCL to macrophages.

***In vivo* therapeutic efficacy on mice with fractures**

To investigate how BCL@MMSNPs-SS-CD-NW affects fracture healing *in vivo*, we intravenously-delivered PBS, BCL@MMSNPs-SS-CD-NW, BCL@MSN-SS-CD-NW, BCL@MSN-SS-CD, or free BCL to a murine fracture model system 1, 4, and 7 days post-injury. The fractures were monitored using X-rays and micro-CT. A significantly reduced fracture gap and a larger callus volume was seen in the mice treated with BCL@MMSNPs-SS-CD-NW, compared to the free BCL group and the other groups. On day 21 post-fracture, the animals treated with BCL@MMSNPs-SS-CD-NW no longer exhibited a clear boundary between the hardened callus and cortical bone, as remodeling had taken place. However, when excluding Fe_3O_4 from the composite BCL@MMSNPs-SS-CD-NW, the beneficial effects of BCL@MMSNPs-SS-CD-NW on fracture healing were partially hindered (**Figure 7A and 7B**). In addition, on day 14, the BCL@MMSNPs-SS-CD-NW-treated mice showed greater total and bone callus volume and BMD compared to other treatments groups, remaining significant on day 21 post-injury (**Figure 7C**). Furthermore, on day 14, bone samples were collected for analysis of M2 macrophage polarization via immunofluorescence, finding that M2 polarization was markedly increased in BCL@MMSNPs-SS-CD-NW-treated animals relative to those in other treatment groups (**Figure 8A-B**). Moreover, the BCL level in the fracture sites was measured by ICP-MS analysis, a relatively high level of BCL was seen in

the BCL@MMSNPs-SS-CD-NW group compared with the other treatment groups (**Figure 8C**). Hematoxylin and eosin (H&E) staining on days 21 further indicated increased bone formation area at the fracture site for mice treated with BCL@MMSNPs-SS-CD-NW, while removal of Fe_3O_4 from the composite impaired this pro-fracture-healing effect (**Figure 8D-E**). This evidence suggests that BCL@MMSNPs-SS-CD-NW effectively accelerates fracture healing in mice.

Discussion

BCL, one of the main ingredients of *Scutellariae radix*, has been clinically used to limit inflammation and is relevant to a wide array of cells related to the healing processes of bone fractures [36]. Additionally, BCL has been shown to protect against MSC apoptosis through anti-oxidation, anti-apoptosis and anti-inflammatory mechanisms [37]. Therefore, BCL has great potential in the prevention and treatment of bone remodeling conditions, including fractures and osteoporosis [38, 39]. However, owing to its low aqueous solubility, its bioavailability is poor, which extensively restricts the bioactivity and clinical use of BCL [40].

The nano-drug delivery system (DDS), however, could solve the problems of BCL mentioned above. The DDS could be easily modified with certain peptide, which has the capability to target the macrophages and subsequently release the BCL by responding to the certain microenvironment. Here, we designed and developed a dual-targeting nanoplatform with magnetic targeting and macrophages targeting. The capacity of magnetic targeting could extremely enhance the accumulation of nanoparticles in the fracture site, which could significantly decrease the side-effect brought by BCL.

Macrophages targeting subsequently regulate the endocytosis of nanoparticles by macrophages.

After entering to the macrophages, the BCL could be released due to the reduction reaction between disulfide bond and GSH. The cleavage of disulfide bond lead to the removal of gatekeeper CD and resulting in the release of BCL. Design and development of dual targeting and GSH response drug release, our nano-platform tackles the problems of poor water solubility and toxic side effects after systemic administration of BCL, which could provide a certain idea for BCL application in the field of fracture treatment clinically.

Elucidating the mechanisms through which BCL affects macrophage phenotype polarization is paramount. Prior studies have reported that BCL has significant roles in the regulation of tumor biology and metastasis [41]. Among the potential regulative mechanisms, BCL-dependent M1 tumor-associated macrophage polarization has been widely demonstrated in various tumor cells, including breast cancer cells and pancreatic cancer cells [42, 43]. Interestingly, a positive correlation between BCL and M2 macrophage polarization has also been reported [44]. In the present study, we found that BCL was able to effectively induce BMDMs towards M2 phenotype polarization, with the ROS/NF- κ B signaling highly involved in this regulative process. We anticipated that treating bone cells with BCL may result in mixed results; BCL inhibits M2 phenotype polarization in cancer cells [45, 46]; while promoting M2

macrophage polarization in a variety of inflammatory diseases [47, 48]. The variable effect of BCL on macrophage polarization requires further investigation in future study. Macrophages have the potential of differentiating into a range of effector subtypes depending on local cellular and molecular signals. The positive regulatory role of M2 macrophages during bone fracture healing is established [49, 50]. Our results demonstrated that BCL@MMSNPs-SS-CD-NW could be delivered to macrophages in a targeted manner, allowing sustained release of BCL. Furthermore, the released BCL effectively induced macrophages towards M2 phenotype polarization, further enhancing osteoblastic differentiation. All these contributed to the beneficial effects of BCL@MMSNPs-SS-CD-NW on bone fracture healing.

The cytotoxicity of BCL has been previously reported in several cells, with evidence suggesting that the release of BCL in the cytosol plays a critical role [51]. In the experiments reported here, we have demonstrated that BCL@MMSNPs-SS-CD-NW could release BCL with the assistance of GSH. To explore the effect of BCL release, we divided BMDMs into 4 groups, treated either with PBS, BCL@MMSNPs-SS-CD-NW, BCL@MMSNPs-SS-CD, or Fe₃O₄@MSN-SS-CD-NW. Assessment with CCK-8, live/dead cell staining, and flow cytometry assays, suggested that free BCL was significantly more toxic than nanoparticle-delivered BCL. Therefore, the released BCL from the BCL@MMSNPs-SS-CD-NW is promising for cell cytotoxicity limitation. We further explored whether the nanoparticles had any effect on the organs. No obvious effects were observed on the H&E stained organs of mice treated with the nanoparticles while obvious lung and liver damage was evident in the organs of mice treated with free BCL.

In the present study, we conducted a two-level targeted nano-system. Firstly, BCL@MMSNPs-SS-CD-NW was accurately delivered to the fracture site using external magnetic field guidance, overcoming the challenge of low concentrations of BCL in the fracture site. The nanoparticle could further influence macrophages through the targeted NW polypeptide. Furthermore, BCL was released from the nanoparticles following interaction with GSH, inducing macrophages towards M2 phenotype polarization. The robust beneficial effects of BCL@MMSNPs-SS-CD-NW on M2 macrophage polarization and bone healing was demonstrated *in vivo*. Taken together, these results indicate that BCL@MMSNPs-SS-CD-NW is a highly safe and effective bone fracture therapy.

We focused on the effect of BCL on macrophages and MSCs. However, BCL may also affect the function of other immune cells and osteoclasts that can potentially regulate the biological processes of bone remodeling. For example, it was recently reported that BCL can regulate the activation of Treg cells in response to inflammation [52]. BCL can also inhibit osteoclast differentiation and induce mature osteoclast apoptosis [53]. Hence, it is of great scientific interest to further explore the effect of our targeted nano-system on other immune cells and osteoclasts during the regenerative processes of bone healing.

Conclusions

Taken together, a dual-targeting nanoplatform based on MMSNPs was designed and developed. Under the stimulation of applied magnetic field at the fracture area, BCL@MMSNPs-SS-CD-NW could be

considerably accumulated in fracture site, which tackles the problem of poor water solubility of BCL and extremely reduces the systemic toxicity of BCL. Subsequently, NW peptide-mediated macrophage endocytosis further improved the biocompatibility of BCL and effectively promoted fracture healing. Furthermore, BCL@MMSNPs-SS-CD-NW presented an excellent biosafety in the mouse fracture model. BCL@MMSNPs-SS-CD-NW efficiently targeted macrophages and polarized them towards the M2 phenotype via suppression of the ROS/NF- κ B pathway. By combining oxidative stress attenuation, effective targeting of macrophages to the fracture site, and timed-release of BCL in a single platform, BCL@MMSNPs-SS-CD-NW demonstrated significant potential for promoting bone reconstruction *in vivo*. In summary, BCL@MMSNPs-SS-CD-NW is a promising therapeutic strategy for bone fracture.

Methods

Materials

Methanol, ethanol, tetraethyl orthosilicate, sodium ascorbate, cuprous bromide and CTAB were provided from Shanghai Chemical Co. (Shanghai, China). Propargyl bromide, mono-6-azido- β -cyclodextrin, 3-mercaptopropyltrimethoxysilane and S-(2-aminoethylthio)-2-thiopyridine hydrochloride were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). The sequence of NW peptide is NWYLPWLGTNDWGGGK(1-AD)-NH₂ and was purchased from Shanghai Hanhong Scientific Co.,Ltd.

Synthesis of MMSNPs

The Fe₃O₄@MSN particles were prepared via a solvothermal method according to the previously reported literature [21, 22]. Briefly, 0.1 g of Fe₃O₄ particles were dispersed in a mixed solution containing of 0.3g CTAB and 80 mL deionized water, 1 g ammonia aqueous solution and 60 mL ethanol. The mixed solution was homogenized for 0.5 h to form a uniform dispersion. 0.40 g of TEOS was added dropwise to the dispersion with continuous stirring. After the reaction for 6 h, the product was collected with a magnet and washed repeatedly with ethanol and water. Finally, the purified particles were redispersed in 60 mL of acetone and refluxed at 80°C for 48 h to remove the template CTAB. The extraction was repeated for three times, and the particles were washed with deionized water, and MMSNPs were finally produced.

Synthesis of MMSNPs-SH

MMSNPs (1.35 g) was dispersed in methanol (108 mL). Then 3-mercaptopropyltrimethoxysilane (3 mL) was added and the mixture was stirred for 24 h at room temperature to obtain MMSNPs-SH. The nanoparticles were then centrifuged (8000 r/min, 10 min), washed six times with methanol and dried under vacuum.

Synthesis of MMSNPs-SS-NH₂

MMSNPs-SH (600 mg) was dispersed in methanol (135 mL). Then S-(2-aminoethylthio)-2-thiopyridine hydrochloride (600 mg) was added. The mixture was stirred at room temperature for 24 h to obtain

MMSNPs-SS-NH₂. The nanoparticles were then centrifuged (8000 r/min, 10 min), washed six times with methanol and dried under vacuum.

Synthesis of MMSNPs-SS-Alkyne

MMSNPs-SS-NH₂ (450 mg) was dispersed in methanol (100 mL) and reaction with propargyl bromide (3 mL) at room temperature for 24 h. The particles was obtained by centrifugation (8000 r/min, 10 min) and washed six times with methanol.

Synthesis of BCL@MMSNPs-SS-CD

MMSNPs-SS-alkyne (100 mg) with 50 mg BCL was dispersed in a mixture of methanol (5 mL) and PBS (5 mL). After stirring at room temperature for 24 h, mono-6-azido- β -CD (100 mg), CuSO₄·5H₂O (100 mg) and sodium ascorbate (170 mg) were added. The mixture was stirred under N₂ atmosphere at room temperature for 3 days to obtain BCL loaded MMSNPs-SS-CD. The nanoparticles were then centrifuged (8000 r/min, 10 min), washed thoroughly with EDTA solution, water and methanol, and dried under vacuum.

Synthesis of BCL@MMSNPs-SS-CD-NW

MMSNPs-SS-CD-NW was prepared through host-guest interaction. MMSNPs-SS-CD (68.5 mg) was dispersed in a mixture of H₂O (7.5 mL) and DMF (2.5 mL). Then NW peptide (140 mg) was added, and the mixture was stirred for 24 h at room temperature to obtain MMSNPs-SS-CD-NW nanoparticles. The nanoparticles were then centrifuged (8000 r/min, 10 min), washed thoroughly with a mixture of water and methanol (V/V = 2/1) and dried under vacuum.

In vitro BCL release

BCL@MMSNPs-SS-CD-NW were dispersed respectively in three different media with pH 7.4, 37 °C: PBS, PBS with 1 mM GSH and PBS with 10 mM GSH. At certain time interval, the nanoparticles have been removed through centrifugation and the concentration of BCL in the medium was analyzed using ultraviolet–visible (UV-vis) absorption spectroscopy at a wavelength of 325 nm.

Culture of macrophages

C57BL/6J mice (average weight: 18g; 6-week-old) from Huazhong University of Science and Technology (HUST) were used for this study. Bone marrow-derived macrophages (BMDMs) were induced using M-CSF as described in a prior study [52]. In brief, bone marrow cells isolated from the mice were cultured in 10% FBS-RPMI-1640 medium (Gibco, Grand Island, USA) containing 50 ng/ml M-CSF (R&D System, Minneapolis, MN, USA) for 7 days. After which the BMDMs were collected for FCM and transcriptomic analysis.

FCM Assay

This was performed as a previous study [52]. BMDMs, M1 macrophages, and M2 macrophages were stained using lineage-specific antibodies, with F4/80 (#123107, Biolegend, San Diego, CA, USA) and CD

209 (#12-2092-80, Sigma–Aldrich) for BMDM identification, F4/80 and CD11c (#117307, Biolegend) for M1 macrophage identification, and F4/80 and CD206 (#141705, Biolegend) for M2 macrophage detection. FlowJo software was used for statistical analysis.

Cell counting kit8 (CCK-8) assay

BMDMs were added to 96-well plates and cultured for 24, or 48 hours respectively. Then, CCK-8 reagent (SigmaAldrich, MO, USA) was added in serum-free medium for 2 hours, followed by measurements of absorbance at 450 nm.

MSC Culture and Transfection

MSCs were also extracted from C57BL/6J mice. Cells were stimulated and cultured in a specific media designed for C57BL/6 mouse mesenchymal stem cells (Cyagen, Guangzhou, China), and used for the *in vitro* assay in three passages.

qRT-PCR

MSCs were treated PBS (PBS group), or co-cultured with M1 macrophages (M1/MSCs group) and M2 macrophages (M2/MSCs group) for 48 hours, and TRIzol® reagent was used to extract total RNA from cells in the different groups. Then, the cDNA reverse transcription process was performed using ReverTra Ace® qPCR RT Master Mix (Toyobo Life Science). The working circumstance of RT process was: 15 minutes at 42°C, followed by 5 minutes at 98°C. GAPDH was served as the internal control and the reaction volume was 20 µL. Relative mRNA levels were calculated according to the $2^{-\Delta\Delta Cq}$ approach and the primer sequences are displayed in Table 1.

Table 1
mRNA primer sequence.

Gene name	Primer sequence (5' to 3')
mmu-Col 1a1-Forward	CTGACTGGAAGAGCGGAGAG
mmu-Col 1a1-Reverse	CGGCTGAGTAGGGAACACAC
mmu-OCN-Forward	TTCTGCTCACTCTGCTGACCC
mmu-OCN-Reverse	CTGATAGCTCGTCACAAGCAGG
mmu-Runx2-Forward	CGCCACCACTCACTACCACAC
mmu-Runx2-Reverse	TGGATTTAATAGCGTGCTGCC
mmu-GAPDH-Forward	AGAGTGTTTCCTCGTCCCG
mmu-GAPDH-Reverse	CCGTTGAATTTGCCGTGA

Western blot

Cells were lysed by the lysis buffer (#AS1004, Aspen, South Africa), and after which the extracted protein was separated via SDS-PAGE and transferred to NC membranes. Next, the membranes were blocked with 5% skim milk and stained overnight at 4°C with antibodies specific for anti-IkB, NIK, p65, pi-p65, pi-IkB (1:500, Abcam, UK), collagen I, ALP, OCN, Runx2 (Abcam, UK), iNOS (1:500, NovusBio, USA, #NB300-605), Arg-1 (1:500, ABclonal, China, #A1847), and β-actin (Abcam, UK, #ab37168). Blots were stained with the according secondary antibodies conjugated to HRP, and proteins were detected with a chemiluminescence examination system. In Fig. 2B and 2E, BMDMs were treated with PBS (Control group), 5 µg/mL BCL, 10 µg/mL BCL, 20 µg/mL BCL, and 40 µg/mL BCL for 48 hours, respectively.

Intracellular ROS detection

The intracellular ROS levels were evaluated by staining with ROS probe dihydroethidium (DHE) (5 µM) for 10 minutes and washed with PBS 3 times. All samples were captured by a microscope (IX53, Olympus, Japan).

Alizarin red staining

MSCs were continuously cultured in 24-well plates in an osteogenic medium (#MUBMX-03011-440, Cyagen, Guangzhou, China) for 21 days to promote osteogenesis. And MSCs were divided into three groups according to the different treatments: MSCs treated with 200 µL PBS was set as PBS group, MSCs co-cultured with M1 macrophages was set as M1/MSCs group, and MSCs co-cultured with M2 macrophages was set as M2/MSCs group. Briefly, cells were washed twice with PBS, after which 10% formalin was added to fix the cells for 15 minutes. 1 mL 0.5% alizarin red staining solution was added to stain the cells at room temperature for 15 minutes. After rinsing with distilled water for 5 minutes, red mineralized nodules were analyzed with a charge-coupled device microscope. Absorbance was measured at 570 nm.

ALP staining

An alkaline phosphatase color development kit (Beyotime Biotechnology, Shanghai, China) was used to conduct the ALP staining of MSCs. And MSCs were divided into 3 groups according to the different treatments: MSCs treated with 200 µL PBS was set as PBS group, MSCs co-cultured with M1 macrophages was set as M1/MSCs group, and MSCs co-cultured with M2 macrophages was set as M2/MSCs group. MSCs were washed twice, and 10% formalin was added to fix the cells for 15 minutes. BCIP/NBT substrate was used to treat cells for 24 hours. Colorimetric changes were analyzed using a charge-coupled microscope and absorbance was measured at 405 nm.

Mice fracture model

We obtained the animal experiments consent from the Institutional Animal Care and Use Committee at Tongji Medical College, HUST. The mice were anesthetized by using pentobarbital sodium (i.p., 50 mg/kg; Sigma-Aldrich), and a model of femoral fracture was constructed via longitudinal incision as we previously reported [52]. The femur was cut by using a diamond disk to yield a mid-diaphysis transverse osteotomy. And then, a 25-gauge intramedullary needle was used to stabilize the fracture site.

ICP-MS analysis

Wage a certain amount of samples into a polytetrafluoroethylene beaker, add 10 mL nitric acid, and heat them in an electric heating plate at 200 °C until solid substances are completely dissolved. If the amount of acid is not enough, continue to add acid in the digestion process. When the digestion solution remains about 1mL, add 1–2 mL of hydrogen peroxide and continue the digestion process. When the solution remains about 1mL, use distilled water to constant volume to 10mL.

And the results were calculated as follow:

$$C_x(mg/kg) = \frac{C_0(mg/L) * f * V_0(mL) * 10^{-3}}{m(g) * 10^{-3}} = \frac{C_1(mg/L) * V_0(mL) * 10^{-3}}{m(g) * 10^{-3}}$$

C_x : Final test results of the element under test, in mg/kg; C_0 : The concentration of the element in the solution is measured in mg/L; f : Dilution ratio; V_0 : After sample digestion, the volume of constant volume, unit is mL; C_1 : The elemental concentration of the sample digestion solution.

In vivo therapy and histochemistry analysis

Mice with fractures were studied in five groups (n = 6 per group) treated with PBS, BCL@MMSNPs-SS-CD-NW (BCL concentration = 20 mg/kg; corresponding particle concentration = 5.3 mg/mL), BCL@MSN-SS-CD-NW (BCL concentration = 20 mg/kg; corresponding particle concentration = 1.0 mg/mL), BCL@MSN-SS-CD (BCL concentration = 20 mg/kg; corresponding particle concentration = 3.9 mg/mL), or Free BCL (BCL concentration = 20 mg/kg) with an external magnetic field. The mice in each of the five groups were injected every two days until completion of the experiments. On day 14, half of the mice were sacrificed and the callus of the fracture sites collected for histology analysis. The remaining mice were sacrificed and the bone tissue collected. The internal organs (heart, liver, spleen, lung and kidney) of the mice were also harvested and used for histology analysis.

X-Rays images

On days 3, 7, 14, and 21 post injury, all animals were subjected to X rays with an In-Vivo FX PRO imaging system (BRUKER, Karlsruhe, Germany) with a 10 second-exposure period.

Micro-computer tomography (Micro-CT) analysis

A micro-CT system (BRUKER 1276, Germany) was taken to image fracture regions in mice, and after scanning, preserved calluses were stored at -80°C prior to further experimental use. Parameters, including bone volume (BV), total volume (TV), BV/TV, and bone mineral density (BMD), were analyzed with a CT Analyser evaluation software (Version: 1.15.4.0) on day 14 and day 21 post-operation. Micro-CT analysis was done in a completely blinded manner with all mice assigned coded sample numbers.

In vivo MRI examination

Fractured mice were intravenously injected with 200 μ L BCL@MMSNP_S-SS-CD-NW (The concentration of BCL was 20 mg/kg and the corresponding particle concentration was 1 mg/mL). Afterwards, the fracture site of the right leg was exposed to an external magnetic field while the left was not. After injection 4, 8, and 12 hours, T2-weighted MR images were recorded with a 7.0 T MR spectrometer (Bruker Inc., Billerica, MA, USA).

Immunofluorescence

On day 14 post-fracture, the callus of the mice was collected and fixed and embedded in an optical cutting temperature compound (Fisher scientific). The embedded tissues were cut to a thickness of 9 μ m using a freezing microtome (LEICA CM-1900, Germany) and permeabilized and blocked with 2% BSA containing 0.5% Triton X-100. The images were taken with a TCS SP5 confocal microscope (LEICA, Germany).

Statistical analysis

Data are presented as means $\pm$ SD. GraphPad Prism 8.0 (GraphPad Software, CA, USA) was used for all statistical analysis. Data were compared with Student's t-tests and one-way ANOVAs with Tukey's post-hoc test as appropriate. $P < 0.05$ was the significance threshold.

Declarations

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Authors' contributions

GL, BM, and WQ designed this study, WZ, LZ, and YX carried out the molecular genetic studies and writing the original draft, HX, WS, TY and LC performed cellular and animal experiments; YH, DX and YS analyzed the data; AP edited and revised the manuscript; FC, GL, LC, and CY provided advice and technical assistance.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to be published.

Declaration of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

Characterization of BCL@MMSNPs-SS-CD-NW. (A) Transmission electron microscope (TEM) image of MMSNPs. (B) hydrodynamic size of the MMSNPs detected by DLS. (C) Zeta potentials of the intermediate products during the preparation process of MMSNPs-SS-CD-NW. (D) BCL release from BCL loaded MMSNPs with different GSH concentrations. (E) Response of MMSNPs to applied magnetic fields. (F) T2-weighted MR images of MMSNPs-SS-CD-NW in aqueous solution at different Fe concentrations.

Figure 2

BCL induces M2 macrophage polarization. (A) The cell morphology of BMDMs. Scale bar = 100 μ m. (B) Identification of the BMDMs proportion from C57BL/6 mice femoral bone marrow via FCM. (C) Phenotypic markers of M1 and M2 macrophages including iNOS and Arg-1 were evaluated Western Blot analysis 48 hours post-treatment. (D-E) The intracellular ROS levels among different groups were assessed by staining with ROS probe dihydroethidium (DHE). (F-G) NIK activation, p65 phosphorylation

and I κ B kinase degradation in BMDMs from BCL-treated groups with different concentrations was studied by Western Blot. Data are mean $\pm$ SD of triplicate experiments. * p <0.05, ** p <0.01, *** p <0.001

Figure 3

M2 macrophage polarization induces MSCs towards osteoblastic differentiation. (A) M1 and M2 macrophages were co-cultured with MSCs in a transwell (membrane pore=0.4 nm) plate. (B) The osteogenic-related markers, including Collagen I, osteocalcin (OCN), and runt-related transcription factor 2 (Runx2), were assessed by Western Blot and (C) qRT-PCR analysis. (D-E) The ALP staining result of MSCs in the different groups. (F-G) The Alizarin Red staining result of MSCs in the different groups. Data are mean $\pm$ SD of triplicate experiments. * p <0.05, ** p <0.01, *** p <0.001.

Figure 4

In vitro biocompatibility of BCL@MMSNPs-SS-CD-NW. (A) CCK-8 assay was used to assess the viability of the cultured BMDMs. (B-C) The apoptosis rate of BMDMs in different groups was assessed using FCM. (D-E) Cell status was checked using live/dead cell staining five days after culture, scale bar = 200 μ m. Data are mean $\pm$ SD of triplicate experiments. * p <0.05, ** p <0.01, *** p <0.001.

Figure 5

Targeted delivery of BCL@MMSNP₃-SS-CD-NW to BMDMs in the fracture site. (A) Fracture sites in right legs were exposed to an external magnetic field, and the fracture sites in left legs were treated as the control group without external magnetic power. (B) The Inductively Coupled Plasma Mass Spectrometry (ICP-MS) result of the Fe³⁺ content in different groups. (C-D) T2 signal intensity suggested that the darkening effect of the fracture sites in right legs was significantly prominent compared with the fracture

sites in left legs. (E) H&E staining of heart, liver, lung, kidney, and spleen of mice in different groups, scale bar = 100 μm . Data are mean $\pm$ SD of triplicate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 6

BCL@MMSNPs-SS-CD-NW induces M2 macrophage polarization. (A-B) The fluorescence intensity in non-blocking group was quantified by FCM. (C-D) The fluorescence intensity in different groups was assessed by FCM. (E) The confocal microscopy image of uptake of labeled BCL@MMSNPs-SS-CD-NW in different groups groups (FITC: Green), scale bar = 30 μm .(F-I) Phenotypic markers of M1 and M2 macrophages including iNOS and Arg-1 in different groups were evaluated by FCM and Western Blot analysis 48 hours post-treatment. Data are mean $\pm$ SD of triplicate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 7

In vivo therapeutic efficacy on mice with fractures. (A) X-rays comparison of fracture healing process among different groups on day 3, 7, 14 and day 21 post-fracture. (B) micro-CT 3D construction, longitudinal sections and cross sections images on post-fracture day 14 and 21. (C) Analysis of BV, TV, BV/TV, BMD of callus among each group. $n = 6$ mice/group, data are mean $\pm$ SD, ** $P < .01$, *** $P < .001$.

Figure 8

Histopathological results of the different groups. (A-B) Immunofluorescence of F4/80 (Green) and Arg-1 (Red) of callus in the different groups, Scale bar = 200 μ m/500 μ m. (C) The BCL level of callus in the different groups was measured by ICP-MS analysis. (D) The Alcian Blue/HE/Orange G staining images and corresponding statistical analysis on day 21 post-injury (scale bar= 500 μ m). Data are mean $\pm$ SD, **P<.01, ***P<.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Scheme1.png](#)
- [Supplementarymaterial.docx](#)