

ACCEPTED PAPER · MAIN TEXT

Core-shell MgO-loaded zinc-crosslinked hydrogel microspheres for immunomodulatory bone regeneration

Paper version: Accepted Paper

Accepted Papers are manuscripts accepted for publication, encompassing all changes made following the peer review process, along with a standard cover page indicating the paper version and an “Accepted Paper” watermark, but excluding any other editing, typesetting or other changes made by AccScience Publishing and/or authors post-acceptance.

Article ID: IJB026380409

Citation: Cao F, Zheng G, Li J, *et al.* Core-shell MgO-loaded zinc-crosslinked hydrogel microspheres for immunomodulatory bone regeneration. *Int J Bioprint.* 2026. doi: 10.36922/IJB026380409

Copyright: © 2026 Author(s). This is an Open Access article distributed under the terms of the Creative Commons Attribution License, permitting distribution, and reproduction in any medium, provided the original work is properly cited.

Publisher’s Note: AccScience Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

AccScience Publishing

RESEARCH ARTICLE

Volume X Issue X (2026)

doi: 10.36922/IJB026380409

Core-shell MgO-loaded zinc-crosslinked hydrogel microspheres for immunomodulatory bone regeneration

Running title: Core-shell MgO@ZnSA/CS hydrogel microspheres for bone regeneration

Fang Cao^{1†}, Guoshuang Zheng^{2†}, Junlei Li¹, Zhihua Cheng¹, Guangxiao Yin¹, Liquan Song¹, Zhijie Ma¹, Jiawei Ying¹, Lu Li¹, Ke Yang³, and Dewei Zhao^{1*}

¹ Department of Orthopedics, Affiliated Zhongshan Hospital of Dalian University, Dalian 116001, China

² Department of Central Laboratory, Affiliated Zhongshan Hospital of Dalian University, Dalian 116001, China

³ Institute of Metal Research, Chinese Academy of Sciences, Shenyang 110016, China

[†]*These authors contributed equally to this work.*

*Corresponding author:

Dewei Zhao (zhaodewei2016@163.com)

Citation: Cao F, Zheng G, Li J, *et al.* Core-shell MgO-loaded zinc-crosslinked hydrogel microspheres for immunomodulatory bone regeneration. *Int J Bioprint.* 2026. doi: 10.36922/IJB026380409

Received: September 16, 2026

Revised: October 13, 2026

Accepted: October 14, 2026

Published online: October 14, 2026

Copyright: © 2026 Author(s). This is an Open Access article distributed under the terms of the Creative Commons Attribution License, permitting distribution, and reproduction in any medium, provided the original work is properly cited.

Publisher's Note: AccScience Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

ACCEPTED PAPER

Abstract

Alginate hydrogel microspheres are attractive as localized ion-delivery matrices for bone regeneration, but rapid ion exchange in physiological media can destabilize the network and produce an early release pulse. Here, MgO-loaded, Zn²⁺-crosslinked alginate cores were coated with chitosan to form core-shell MgO@ZnSA/CS hydrogel microspheres by electrospraying and electrostatic deposition. The resulting polyelectrolyte-complex shell reduced swelling, slowed degradation, and prolonged Mg²⁺ and Zn²⁺ release while providing a cell-adhesive interface. Two MgO contents were evaluated alongside Ca-crosslinked and Zn-crosslinked controls. *In vitro*, all formulations supported bone marrow mesenchymal stem cell viability. The low-MgO formulation (LoMgO@ZnMs) produced the greatest alkaline phosphatase staining, matrix mineralization, Runx2 expression, and osteogenic gene expression among the tested groups. In RAW264.7 macrophages cultured without inflammatory prestimulation, Zn-containing formulations increased Arg-1 and *Il4/Il10* and reduced iNOS and *Il6/Tnfa*, consistent with a shift toward a pro-regenerative marker profile; LoMgO@ZnMs generally showed the strongest response. In a rat critical-sized calvarial defect model, LoMgO@ZnMs generated the highest new-bone indices and the most continuous mineralized tissue after 8 weeks. These findings identify chitosan-shelled, MgO-loaded zinc-alginate hydrogel microspheres as a sustained ion-delivery platform with dose-dependent osteogenic and immunomodulatory activity for non-load-bearing bone regeneration.

Keywords: Alginate hydrogel Microspheres; Chitosan; Magnesium oxide nanoparticles; Zinc crosslinking; Sustained ion release; Osteoimmunomodulation; Bone regeneration

1. Introduction

Critical-sized bone defects caused by trauma, tumor resection, or infection remain difficult to repair and motivate the development of biomaterials that can support both early host responses and subsequent bone formation ^[1]. The immune environment established after implantation influences the trajectory of tissue regeneration ^[2]. Macrophages are highly plastic innate immune cells, and changes in their inflammatory and pro-regenerative marker profiles can affect the recruitment, survival, and osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) ^[3].

Alginate-based hydrogels are widely investigated for bone tissue engineering because their hydrophilic polymer networks can be formed under mild conditions, shaped into injectable microspheres, and tuned through ionic crosslinking ^[4]. Calcium-crosslinked alginate microspheres are established carriers but provide limited intrinsic biological signaling ^[5]. Zn^{2+} is an essential trace element in bone metabolism and a catalytic cofactor for alkaline phosphatase (ALP), an enzyme involved in matrix mineralization ^[6,7]. Zinc-containing biomaterials have also been associated with pro-regenerative macrophage responses ^[8]. Thus, using Zn^{2+} as both an alginate crosslinker and a released bioactive ion may integrate hydrogel network formation with local biological activity.

For bone tissue engineering, the principal advantages of hydrogels are their water-rich, extracellular-matrix-like environment, mild processing conditions, conformability to irregular defects, injectability, and capacity for localized delivery of cells or soluble bioactive cues. In microsphere form, they additionally provide a high surface area and modular building blocks that can fill non-load-bearing defects or be incorporated into reinforced constructs. Their high water content and compliant polymer networks, however, generally provide insufficient stiffness and structural stability for load-bearing repair when used alone. Accordingly, the microspheres investigated here were designed as an injectable local ion-delivery component rather than as a stand-alone load-bearing implant.

Ionically crosslinked alginate networks can nevertheless swell, exchange crosslinking ions, and lose structural integrity in phosphate-containing physiological fluids, which may produce an undesirable early release pulse ^[9]. To address this limitation, we designed a Zn^{2+} -crosslinked alginate hydrogel core surrounded by a chitosan (CS) shell. Electrostatic complexation between protonated CS amino groups and alginate carboxyl groups was expected to form a polyelectrolyte-

complex (PEC) interface that limits water penetration and outward ion diffusion. MgO nanoparticles were incorporated into the hydrogel core as an additional magnesium source because magnesium-containing biomaterials can support osteogenic and immunomodulatory responses [10,11]. We therefore hypothesized that the core-shell architecture would regulate Mg^{2+} and Zn^{2+} release and improve the biological performance of the hydrogel microspheres.

Here, core-shell MgO@ZnSA/CS hydrogel microspheres with two MgO loading levels (LoMgO@ZnMs and HiMgO@ZnMs) were fabricated and characterized. Swelling, degradation, ion release, surface structure, BMSC responses, and RAW264.7 macrophage marker profiles were evaluated *in vitro*. Bone-regenerative performance was further assessed in a rat critical-sized calvarial defect model using micro-computed tomography (micro-CT), histology, and immunohistochemistry. The study was designed to determine how the chitosan shell and MgO loading influence the physicochemical and biological behavior of zinc-crosslinked alginate hydrogel microspheres.

2. Materials and methods

2.1. Preparation of ionically crosslinked core-shell hydrogel microspheres

Sodium alginate (SA; Mw = 530 kDa, viscosity = 100 cp) was slowly sprinkled into ultrapure water under magnetic stirring at 200 rpm at room temperature and continuously stirred for 4 h to prepare a 1.5% (w/v) homogeneous solution. Nano-MgO powder was added to achieve final MgO molar concentrations of 1, 5, 10, and 20 mM, followed by sonication for 15 min for uniform dispersion. The microspheres were fabricated using an electrostatic droplet generator (YD-04, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China). Each precursor solution was delivered through the needle using a syringe pump at a constant flow rate of 20 mL/h. During microsphere generation, the applied voltage and pulse frequency were maintained at 50 kV and 120 Hz, respectively, and the distance between the needle tip and the surface of the collection bath was fixed at 2 cm. The generated droplets were collected in 1.5% (w/v) CaCl_2 or ZnCl_2 gelling bath to form hydrogel microspheres.

Fresh wet microspheres were rinsed 2–3 times with ultrapure water to remove residual free ions and reduce chitosan– Zn^{2+} aggregate formation, then immersed in 1.0% (w/v) chitosan (CS) solution (deacetylation degree = 85%, Mw = 38 kDa; dissolved in 0.1 M acetic acid, pH 4.5–5.0). Under this acidic environment, protonation of amino groups on chitosan chains provided positive charges

for electrostatic interaction with negatively charged zinc–alginate microspheres. Coating was performed at 100 rpm for 10 min at room temperature to form core-shell microspheres via electrostatic adsorption. After coating, microspheres were washed with physiological saline to terminate adsorption and remove unbound chitosan. Rhodamine-labeled chitosan confirmed the uniform and continuous shell structure. Following preliminary cytocompatibility screening, 5 mM and 10 mM MgO-loaded formulations were selected as the low-dose and high-dose groups for subsequent experiments, designated as LoMgO@ZnMs and HiMgO@ZnMs, respectively. Of note, zinc-alginate/chitosan core-shell microspheres without MgO loading are abbreviated as ZnMs throughout the manuscript.

2.2. Physicochemical characterization of hydrogel microspheres

Morphology and particle size were observed by optical microscopy, with diameters of at least 50 individual microspheres per group quantified via Image-Pro Plus v6. Functional groups were identified by FTIR (Nicolet iS-50, Thermo Scientific). Surface microstructure was analyzed by SEM (SU3500, Hitachi) at 10 kV after gold sputtering; elemental composition and distribution were detected by EDS (EDAX Element System, USA) with mapping of C, O, Ca, Zn and Mg. For swelling tests, 5 mg microspheres were submerged in 5 mL PBS. Wet weight was measured at preset time points, and swelling ratio was calculated as $(W_t - W_0)/W_0 \times 100\%$ (W_t = wet weight, W_0 = initial dry weight). For degradation assays, 5 mg dry microspheres were incubated in 10 mL PBS at 37 °C; residual dry weight (W_t) was recorded at each scheduled week after lyophilization. The remaining weight percentage was calculated as $W_t/W_0 \times 100\%$ (W_0 = initial dry weight). Supernatants were collected at scheduled time points for Mg^{2+}/Zn^{2+} quantification via ICP-OES (5110, Agilent), with an equal volume of fresh PBS replenished after each collection.

2.3. Cell culture and cytocompatibility

Mouse BMSCs (M131) were cultured in DMEM (Cytiva, US) containing 10% FBS (Gibco, US) and 1% penicillin-streptomycin (Coolaber, China). RAW264.7 received high-glucose DMEM with the same serum and antibiotic supplements. Sterilized microspheres were placed in 96-well plates, with 5×10^3 BMSCs seeded per well, cells without microspheres served as control. Cell proliferation was detected by CCK-8 kit (Dojindo, Japan) on days 1, 4 and 7. After 3 days of co-

culture, Live/Dead staining (Calcein AM/PI, Yeason, China) was performed, and images were captured by CLSM (LSM9, Zeiss).

2.4. Cell adhesion

BMSCs co-cultured with microspheres for 3 days were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin (BSA). F-actin and nuclei were stained with rhodamine-conjugated phalloidin and DAPI, respectively, and observed by CLSM. For SEM observation, samples were fixed with 2.5% glutaraldehyde, dehydrated with gradient ethanol, freeze-dried, gold-sputtered, and examined at 5 kV.

2.5. Osteogenic differentiation assays

Osteogenic differentiation was evaluated using Transwell inserts (0.4 μ m pore size). BMSCs (5×10^4 cells/well) were seeded in the lower chamber of 24-well plates. After adhesion, medium was replaced with osteogenic induction medium, and sterilized microspheres were placed in the upper inserts; wells without microspheres served as control. ALP staining was performed on day 14, and Alizarin Red S (ARS) staining on day 21, positive areas were quantified by Image-Pro Plus v6. Immunofluorescence staining of Runx2 (1:500, ab192256, Abcam) was conducted on day 7. After washing, cells were incubated with secondary antibody (ab150077, 1:500, Abcam, UK) for 60 min. Cytoskeletal F-actin and nuclei were stained with iFluor 594-conjugated phalloidin (Yeasen, China) and DAPI, respectively. Images were captured with CLSM (LSM9, Zeiss, Germany).

Total RNA was isolated with Takara RNAiso reagent. RNA concentration and purity were measured via a NanoDrop spectrophotometer (Thermo Fisher, US). Following reverse transcription using a Takara reverse transcription kit, qPCR amplification was conducted on a 7500 Fast System (Thermo Fisher, US) with SYBR Green Master Mix. The relative expression of *Alp*, *Ocn*, *Col1a1* and *Runx2* was normalized against *Gapdh* and quantified using the $2^{-\Delta\Delta Ct}$ method. Full primer information is provided in Table S1.

2.6. Macrophage marker analysis

RAW264.7 cells (1×10^5 cells/well) were seeded in the lower chamber, with microspheres in the upper inserts. Immunofluorescence staining of iNOS (1:500, 18985-1-AP, Proteintech) and Arg-1 (1:500, 16001-1-AP, Proteintech) was performed as described above. mRNA levels of M1-related (*Il6*, *Tnfa*) and M2-related (*Il4*, *Il10*) genes were detected by qRT-PCR, with primers listed in Table S1.

2.7. Rat critical-sized calvarial defect model

Sprague-Dawley rats ($n = 20$, 8–10 weeks, 250–300 g, male/female = 1:1) were used, with animal experiments approved by the Laboratory Animal Welfare and Ethics Committee of Dalian University Affiliated Zhongshan Hospital (No. DW2025-028-01). Rats were randomly divided into 5 groups ($n = 4$): blank control (5 mm calvarial defect without implantation), CaMs, ZnMs, LoMgO@ZnMs, and HiMgO@ZnMs. Rats were anesthetized through intraperitoneal injection of sodium pentobarbital dissolved in sterile normal saline (40 mg/kg). A 5 mm defect was drilled using a high-speed dental drill (S2 Mini, Stryker, US). During drilling, the surgical site was continuously irrigated with sterile saline to cool the bone tissue and prevent thermal osteonecrosis. Sterilized microspheres were injected into the bone defect cavity using a syringe, with a fixed filling volume of 25 mm³ microspheres for each defect; the control group received no material implantation. Subsequently, the periosteum and skin were sutured sequentially. Postoperative intramuscular penicillin administration was performed to prevent surgical site infection. At 8 weeks after implantation, the rats were euthanized sequentially to collect cranial bone specimens for subsequent detection.

2.8. Micro-CT and histological assessments

Calvarial specimens were scanned by micro-CT (Inveon, Siemens) for 3D reconstruction. A cylindrical ROI (5 mm diameter, 2 mm height) was defined to quantify bone parameters: bone volume/total volume (BV/TV), bone surface/bone volume (BS/BV), trabecular thickness (Tb.Th), and trabecular number (Tb.N), which were quantitatively assessed using the accompanying analysis software. Samples were decalcified in EDTA, dehydrated, embedded, and sectioned (RM2235, Leica, Germany) at the defect center. Hematoxylin & eosin (HE) and Masson's trichrome staining were performed to evaluate tissue morphology, inflammatory infiltration, and newly formed bone tissue within the defect area.

2.9. Immunohistochemical analysis

IHC staining was performed on serial paraffin sections to detect ALP (1:500, PA5-106391, Invitrogen), OCN (1:500, 33-5400, Invitrogen), CD86 (1:200, GB150054, Servicebio) and Arg-1 (1:100, GB115724, Servicebio). Following dewaxing, antigen retrieval and blocking, sections were incubated with primary antibodies overnight, then with HRP-labeled secondary antibodies

for 1 h. Positive signals were visualized with DAB kit, and positive area percentages were quantified via Saiviewer software.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism 11.0 (GraphPad Software, Boston, MA, USA). Data are presented as mean $\pm$ standard deviation (SD). Unless otherwise stated, $n = 3$ for *in vitro* biological replicates and $n = 4$ animals per group for *in vivo* quantitative analyses. Normality and homogeneity of variance were assessed before parametric testing. Comparisons among multiple groups at a single time point were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Time-course data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison test. A two-sided p value < 0.05 was considered statistically significant. Significance levels are indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results

3.1. Fabrication and morphology of core-shell hydrogel microspheres

Core-shell hydrogel microspheres were fabricated by electrospraying alginate into divalent-cation gelling baths followed by electrostatic deposition of CS (Figure 1A). All formulations showed uniform spherical morphology under optical microscopy (Figure 1B). CLSM was used to examine the spatial distribution of rhodamine-labeled CS (Figure 1C). Rhodamine-labeled CS exhibited uniform and continuous fluorescent signals along the periphery of the microspheres without obvious aggregation, and the hydrated CS shell maintained a consistent thickness of 10–15 μm . Particle-size analysis showed relatively narrow distributions for all groups (Figure 1D). The mean diameters of LoMgO@ZnMs and HiMgO@ZnMs were 193.14 and 181.75 micrometers, respectively, compared with 178.25 micrometers for ZnMs and 179.95 micrometers for CaMs.

3.2. Chemical composition and network interactions

FTIR spectra supported PEC formation between SA and CS: the overlapping O–H/N–H band at 3200–3600 cm^{-1} was consistent with intermolecular hydrogen bonding, while the shift of the COO^- band from 1633 to 1620 cm^{-1} was consistent with electrostatic interactions between alginate carboxyl groups and CS amino groups (Figure 1E). The main polysaccharide bands remained detectable after processing. Shifts in the carboxylate region and the band near 541 cm^{-1} were

consistent with Zn^{2+} -alginate coordination, while the band near 419 cm^{-1} supported MgO incorporation and increased with the nominal MgO loading.

3.3. Swelling, degradation, and ion-release behavior

Bare Zn^{2+} -crosslinked alginate hydrogel microspheres without a CS coating were included as an additional control in the swelling and ion-release experiments to examine the contribution of the shell. All groups approached swelling equilibrium within 12 h. CS-coated formulations displayed lower equilibrium swelling than bare ZnSA microspheres, consistent with restriction of water uptake by the interfacial PEC layer (Figure 1F).

All formulations underwent gradual mass loss over 8 weeks (Figure 1G). Bare ZnSA microspheres showed greater early mass loss, whereas the CS-coated formulations retained mass for longer, indicating that the coating improved stability in PBS. The CS-coated microspheres were nearly degraded by week 8.

Mg^{2+} and Zn^{2+} release showed an initial faster phase during the first 5 days followed by continued release through day 21 (Figures 1H and 1I). Cumulative Mg^{2+} release reached 78.51% for LoMgO@ZnMs and 82.89% for HiMgO@ZnMs on day 21. Bare ZnSA released Zn^{2+} most rapidly, reaching 75.90% by day 9, while the CS-coated formulations reached 69.22-75.65% by day 21. These comparisons support the role of the CS-rich shell as a diffusion barrier that moderates the early release phase and extends ion delivery.

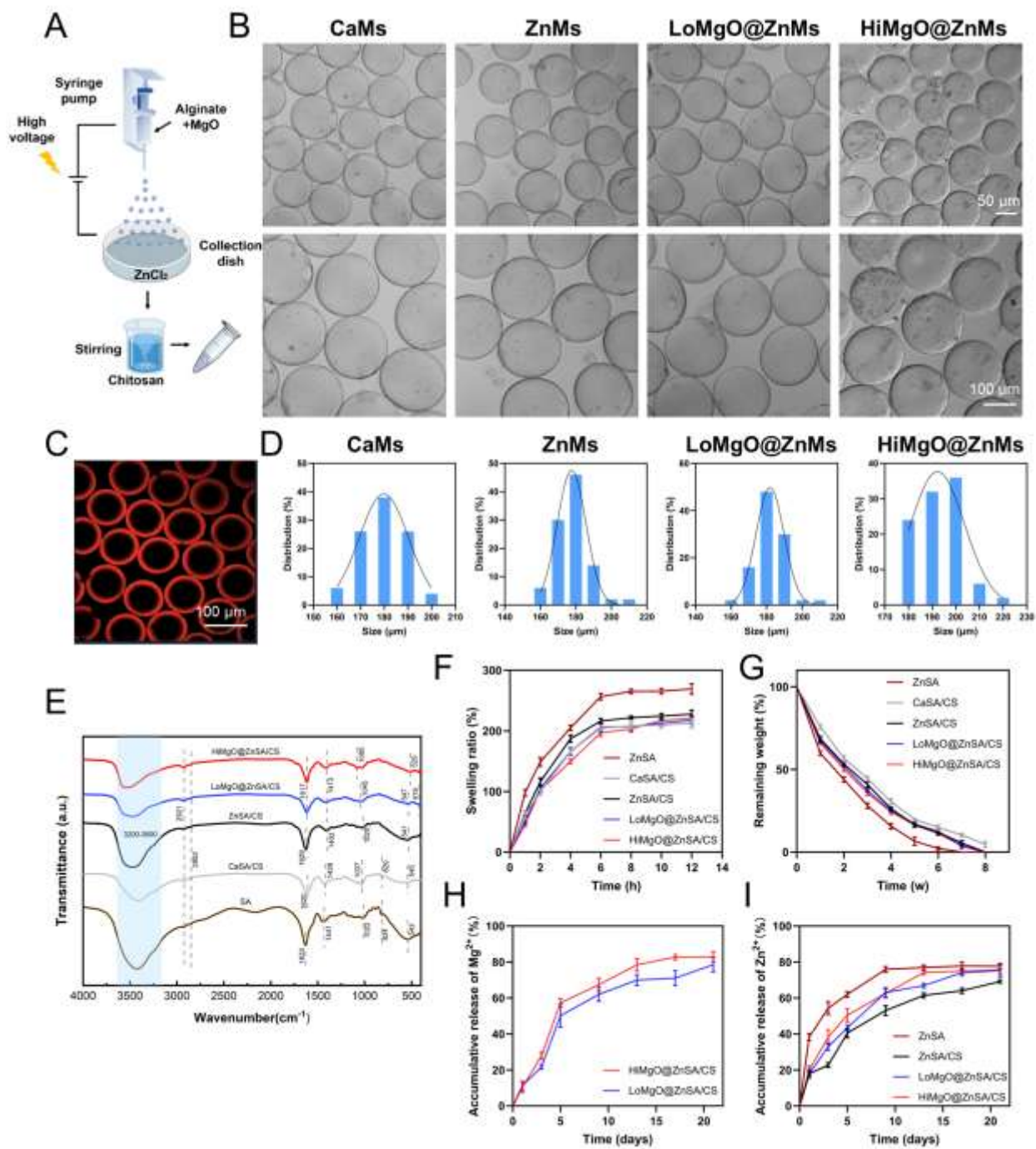


Figure 1. Fabrication and physicochemical analysis of multifunctional composite microspheres. (A) Schematic illustration of the electrospray fabrication and CS coating process for composite microspheres. (B) Representative optical microscopy images and size distribution of CaMs, ZnMs, LoMgO@ZnMs, and HiMgO@ZnMs (scale bar: 50 and 100 μm). (C) CLSM image confirming the core-shell architecture with red fluorescence representing the rhodamine-labeled CS shell (scale bar: 100 μm). (D) Particle size distribution histograms of the corresponding groups, indicating a narrow size range centered around 180–200 μm . (E) FTIR spectra of SA, CaSA/CS, ZnSA/CS, and MgO-loaded microspheres, illustrating the successful crosslinking and polyelectrolyte complexation. (F) Swelling ratios and (G) *In vitro* degradation profiles (weight remaining %) of the microspheres. (H) Cumulative Mg^{2+} and (I) Zn^{2+} release profiles for 21 days.

3.4. Surface topography and elemental distribution

SEM showed that the lyophilized microspheres retained an overall spherical geometry with wrinkled surfaces (Figure 2). CaMs appeared relatively smooth and compact, whereas ZnMs displayed a more folded surface. MgO-loaded formulations showed increasingly rugged surfaces with increasing nominal MgO content.

EDS confirmed the presence of C, O and corresponding crosslinking ions (Ca^{2+} or Zn^{2+}) in each group. Mg signals were detected in MgO-loaded formulations, with intensity positively correlated to loading dose. Elemental mapping revealed uniform distribution of Zn and Mg throughout the microsphere matrix without obvious aggregation.

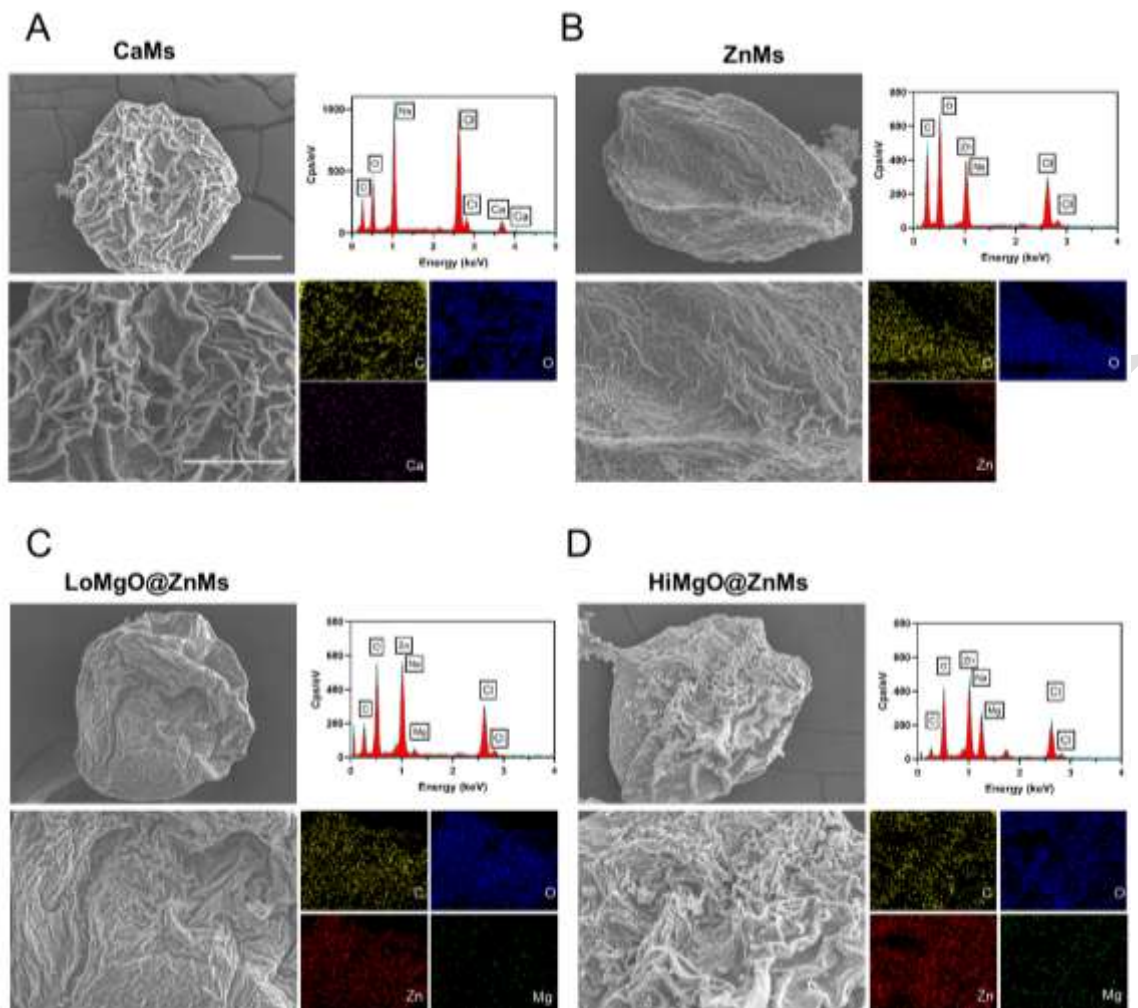


Figure 2. Morphological and elemental characterization of hydrogel microspheres. (A–D) SEM images, high-magnification surface topographies, EDS spectra, and corresponding elemental mapping results of (A) CaMs, (B) ZnMs, (C) LoMgO@ZnMs, and (D) HiMgO@ZnMs. SEM images indicated that all groups kept a general spherical geometry with a characteristic wrinkled surface caused by the sublimation of ice crystals during the lyophilization process. The EDS spectra and elemental mapping images confirmed the successful and homogeneous incorporation of crosslinking ions (Ca^{2+} or Zn^{2+}) and MgO nanoparticles (Mg^{2+}) in the alginate-CS framework. The co-localization of C, O, Zn, and Mg signals in the MgO-loaded groups confirmed the successful encapsulation of bioactive components inside the composite microspheres. Scale bar in (A): 50 μm .

3.5. Cell adhesion and cytocompatibility

All microsphere formulations showed favorable cytocompatibility without an obvious increase in dead cells; proliferation profiles and Live/Dead images are provided in Figure S1. High-magnification SEM and F-actin staining were used to examine cell-material interactions (Figure 3). Bare ZnSA hydrogel microspheres were included as an additional cell-adhesion control. Compared with the relatively rounded cells observed on bare ZnSA, BMSCs associated with ZnMs and MgO@ZnMs displayed a more elongated and spread morphology. Cells in the MgO-loaded

groups showed organized cytoskeletal structures and multiple protrusions in contact with the wrinkled microsphere surface (Figure 3B). These observations indicate that the CS-coated surface supported BMSC attachment and spreading.

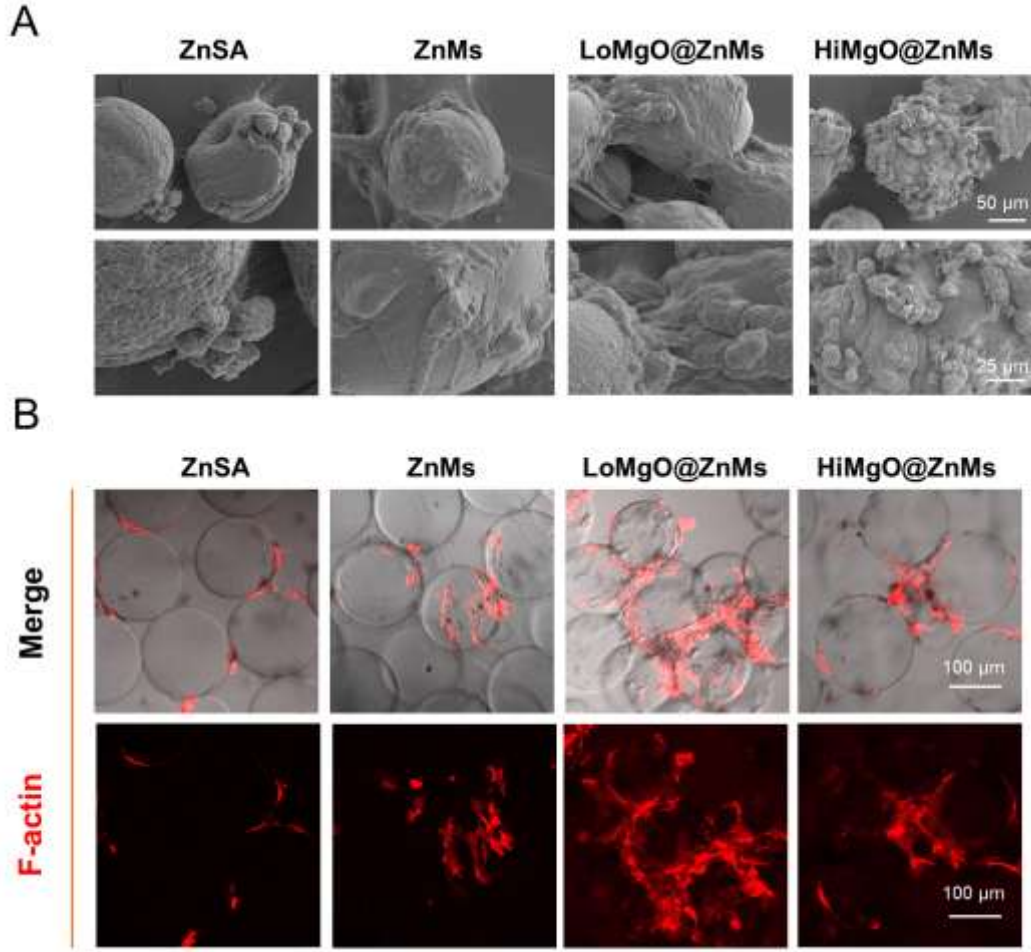


Figure 3. Cell adhesion and morphological characterization on microspheres. (A) SEM images revealing the cell adhesion and spreading on the wrinkled surfaces of ZnSA, ZnMs, and MgO-loaded microspheres. (B) F-actin staining (red) and merged fluorescence images illustrating the formation of extensive filopodia and lamellipodia in the MgO-loaded groups. Scale bars: 50 and 25 μm for (A); 100 μm for (B).

3.6. Osteogenic differentiation

ALP and Alizarin Red S (ARS) staining were performed on days 14 and 21, respectively (Figure 4A). Microsphere-treated groups showed stronger ALP staining and more mineralized nodules than the untreated control. LoMgO@ZnMs produced the largest ALP-positive area and the most extensive mineral deposition, whereas HiMgO@ZnMs showed a smaller response. CaMs and

ZnMs produced comparable osteogenic responses that were greater than the control. LoMgO@ZnMs exceeded CaMs and ZnMs in both assays and also exceeded HiMgO@ZnMs in ALP-positive area ($p < 0.05$), demonstrating an MgO-loading-dependent effect, with the lower tested loading producing the stronger response within the tested zinc-crosslinked formulations (Figures 4C and 4D).

Runx2 immunofluorescence supported the staining results (Figures 4B and 4I). LoMgO@ZnMs produced the highest Runx2 fluorescence intensity, exceeding ZnMs ($p < 0.001$) and HiMgO@ZnMs ($p < 0.05$). qRT-PCR also showed increased expression of osteogenic markers in the MgO-loaded groups. LoMgO@ZnMs generally produced the highest expression; *Alp*, *Col1a1*, and *Runx2* were higher than in ZnMs and HiMgO@ZnMs ($p < 0.001$ for the reported comparisons; Figures 4E-4H). These data indicate that MgO loading altered the osteogenic activity of the zinc-crosslinked hydrogel microspheres, with the lower tested loading providing the strongest response.

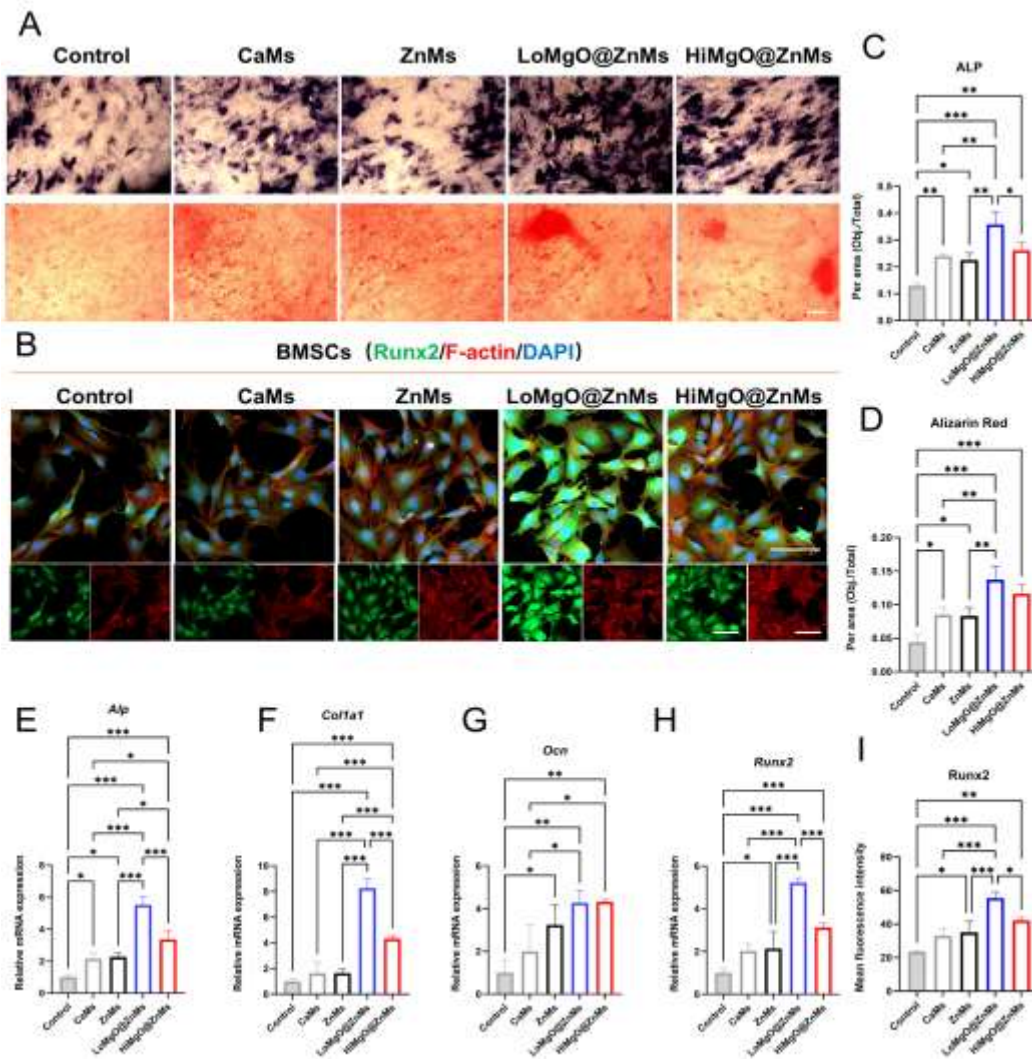


Figure 4. Osteogenic differentiation of BMSCs induced by microspheres. (A) Representative images of ALP staining and Alizarin Red S staining cultured with different microsphere groups. (B) Immunofluorescence staining of Runx2 (green), F-actin (red), and DAPI (blue) in BMSCs after 7 days of induction. (C, D) ALP-positive areas and mineralized nodule areas normalized to the total area. (E–H) qRT-PCR analysis of Alp, Col1a1, Ocn, and Runx2. (I) Mean fluorescence intensity for Runx2 corresponding to the images in (B). Data are presented as mean $\pm$ SD ($n = 3$). Scale bars: 100 μ m. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.7. Macrophage marker responses

Immunomodulatory responses were evaluated in RAW264.7 macrophages cultured with the microspheres in the absence of inflammatory prestimulation. Arg-1 fluorescence was higher in the ZnMs group than in the control and CaMs groups ($p < 0.001$), while iNOS was lower than in CaMs ($p < 0.05$). LoMgO@ZnMs produced the highest Arg-1 signal and the lowest iNOS signal among

the tested groups; Arg-1 intensity was higher than in ZnMs ($p < 0.01$; Figures 5A, 5C, and 5D). These marker changes are consistent with a more pro-regenerative macrophage phenotype but do not establish reversal of a pre-existing inflammatory state.

qRT-PCR showed a similar pattern (Figures 5E-5H). CaMs produced little change in the measured inflammation-related transcripts. ZnMs, LoMgO@ZnMs, and HiMgO@ZnMs increased *Il4* and *Il10* and reduced *Il6* and *Tnfa* relative to the control and CaMs groups. LoMgO@ZnMs generally produced the largest changes: *Il4* and *Il10* were higher than in ZnMs, and *Il10* was also higher than in HiMgO@ZnMs. *Il6* and *Tnfa* were reduced relative to the control and CaMs groups, and *Tnfa* was lower than in ZnMs. Together, the protein and transcript data indicate a MgO-content-dependent shift in macrophage marker expression, with LoMgO@ZnMs producing the strongest pro-regenerative profile under the tested basal culture conditions.

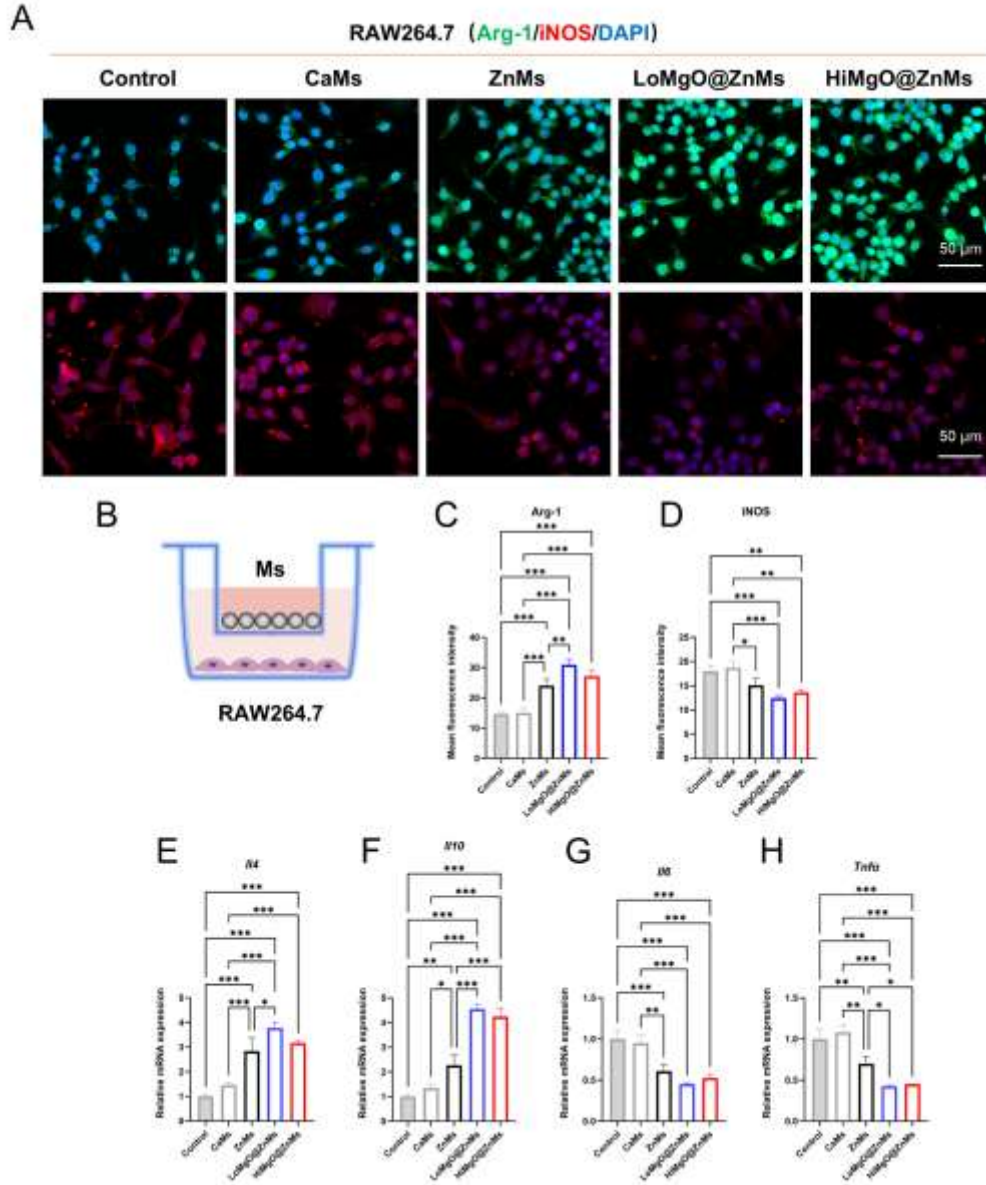


Figure 5. Macrophage marker responses to hydrogel microspheres. (A) Immunofluorescence images of RAW264.7 macrophages after 3 days of co-culture showing Arg-1 (green), iNOS (red), and nuclei (DAPI, blue). (B) Schematic of the Transwell co-culture configuration. (C, D) Quantification of Arg-1 and iNOS mean fluorescence intensity. (E–H) Relative expression of *Il4*, *Il10*, *Il6*, and *Tnfa*. Cells were not prestimulated with LPS. Data are presented as mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bars: 50 μ m.

3.8. Micro-CT evaluation *in vivo*

Micro-CT demonstrated group-dependent bone regeneration (Figure 6A, 6D–G). At week 8, LoMgO@ZnMs showed the most extensive mineralized bridging across the defect, followed by HiMgO@ZnMs, ZnMs, and CaMs. The LoMgO@ZnMs group exhibited the highest BV/TV, which was significantly greater than those of the Control, CaMs, ZnMs, and HiMgO@ZnMs

groups ($p < 0.001$). BV/TV was also higher in the HiMgO@ZnMs group than in the Control ($p < 0.01$), CaMs ($p < 0.05$), and ZnMs ($p < 0.01$) groups. For BS/BV, the CaMs group showed significantly higher values than the ZnMs and HiMgO@ZnMs groups ($p < 0.05$), whereas no other significant differences were indicated. Compared with the Control group, Tb.Th was significantly higher in the ZnMs and LoMgO@ZnMs groups ($p < 0.05$) and in the HiMgO@ZnMs group ($p < 0.01$). Compared with the CaMs group, the ZnMs, LoMgO@ZnMs, and HiMgO@ZnMs groups all exhibited significantly greater Tb.Th ($p < 0.01$). The LoMgO@ZnMs group also exhibited the highest Tb.N, with significantly greater values than all other groups ($p < 0.001$). In addition, Tb.N was higher in the CaMs and HiMgO@ZnMs groups than in the Control ($p < 0.05$) and ZnMs ($p < 0.01$) groups.

3.9. Histological evaluation *in vivo*

HE and Masson's staining of 8-week calvarial sagittal sections (Figure 6B, C) were applied to evaluate bone regeneration, collagen deposition and local tissue morphology. Under low magnification, the control group showed negligible new bone formation, with defects mainly filled by loose fibrous connective tissue. The CaMs and ZnMs groups formed limited new bone at defect margins, leaving obvious central gaps. The LoMgO@ZnMs group achieved nearly complete defect bridging with continuous bone tissue, while the HiMgO@ZnMs group exhibited moderate regeneration inferior to the LoMgO@ZnMs group. High-magnification views revealed no mineralized matrix and sparse fibroblasts in controls, and scattered immature osteoid at margins in the CaMs and ZnMs group. The LoMgO@ZnMs group presented abundant ordered osteoblasts, dense bone matrix and mild inflammatory infiltration, whereas the HiMgO@ZnMs group formed immature trabeculae.

Masson's trichrome staining showed the broadest mineralized matrix distribution in the LoMgO@ZnMs group, whereas the control, CaMs, and ZnMs groups showed weaker and more localized staining. High-magnification images showed thicker and more interconnected trabeculae with LoMgO@ZnMs than in the other groups. These histological observations were consistent with the micro-CT measurements and support the superior regenerative performance of the lower MgO loading among the formulations tested.

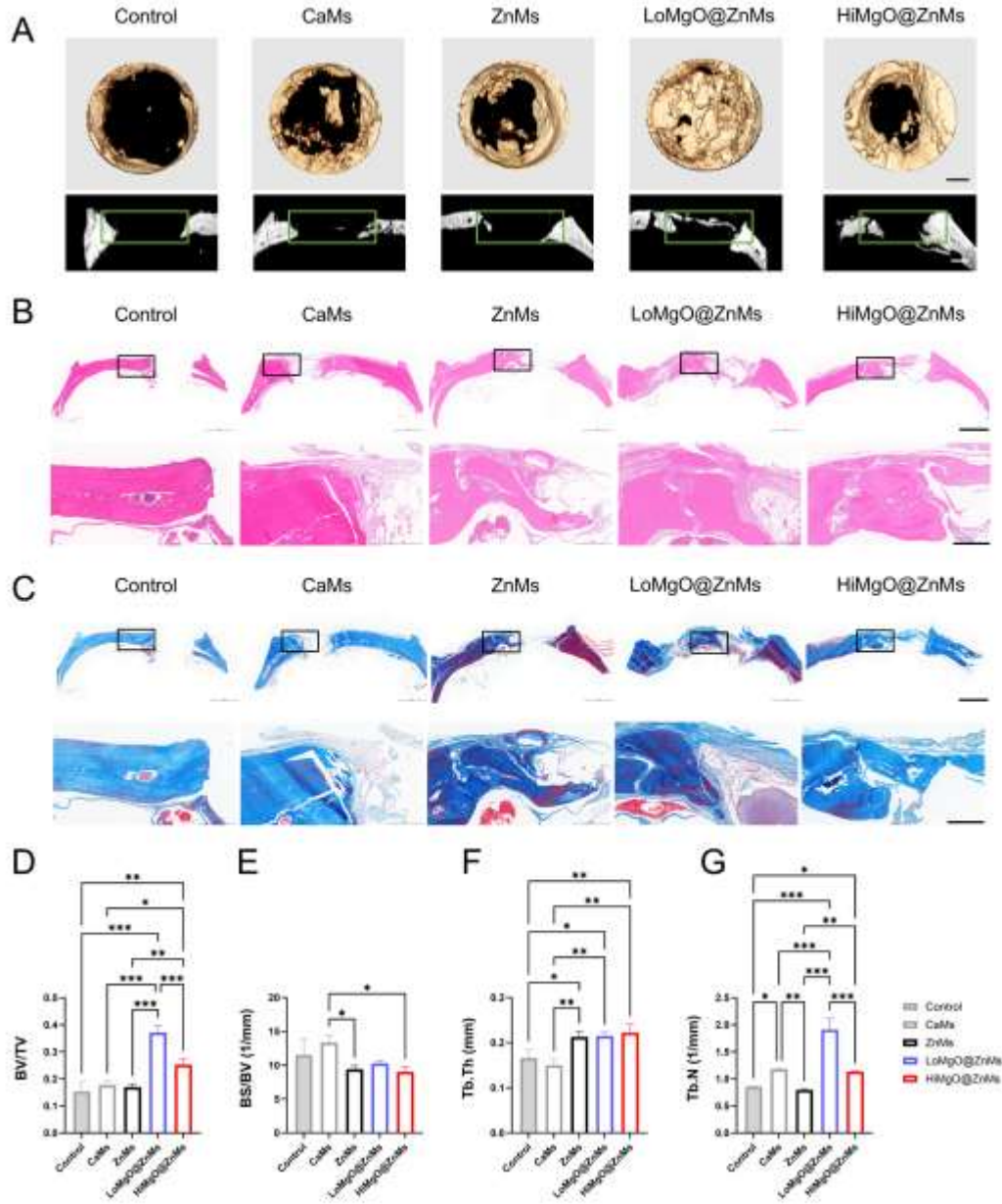


Figure 6. *In vivo* bone regeneration evaluation. (A) 3D reconstruction and 2D images of bone defects in Control, CaMs, ZnMs, LoMgO@ZnMs, and HiMgO@ZnMs groups. (B, C) Panoramic and magnified histological images stained with HE (B) and Masson (C), respectively. (D-G) Quantitative analysis of BV/TV, BS/BV, Tb.Th and Tb.N. Data are represented as mean $\pm$ SD ($n = 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bars: 1 mm for (A); 2 mm and 500 μ m for (B) and (C). The rectangular boxes indicate the calvarial defect regions selected for micro-CT and histological evaluation.

3.10. Immunohistochemical evaluation *in vivo*

For the osteogenic markers ALP and OCN (Figure 7), LoMgO@ZnMs showed the largest positive areas among the tested groups. HiMgO@ZnMs also showed greater staining than the control,

CaMs, and ZnMs groups but remained below LoMgO@ZnMs. The in situ protein-expression pattern was therefore consistent with the micro-CT and histological outcomes.

CD86 and Arg-1 immunohistochemistry was used to characterize local macrophage-associated marker expression (Figure 8). The control and CaMs groups showed greater CD86 staining and limited Arg-1 staining. Zinc-containing hydrogel microspheres reduced the CD86-positive area and increased the Arg-1-positive area. LoMgO@ZnMs showed the lowest CD86 signal and the highest Arg-1-positive proportion; Arg-1 staining was higher than in the control, CaMs ($p < 0.001$), and ZnMs ($p < 0.01$) groups. Arg-1 did not differ significantly between LoMgO@ZnMs and HiMgO@ZnMs.

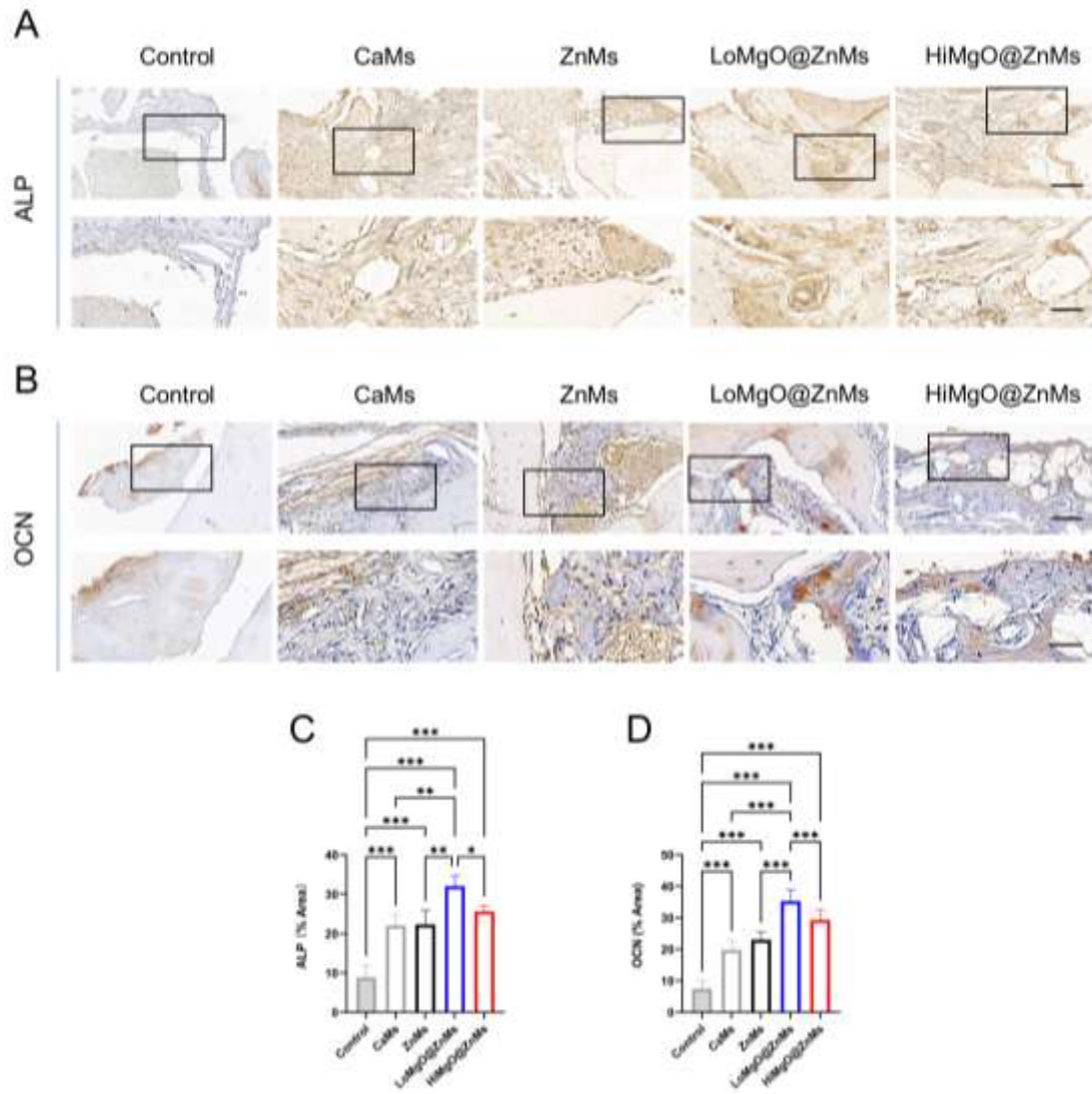


Figure 7. Immunohistochemical detection and quantitative statistics of osteogenic ALP and OCN protein expression at 8 weeks after implantation. (A, B) Panoramic and magnified immunohistochemical images of ALP (A) and OCN (B) in Control, CaMs, ZnMs, LoMgO@ZnMs, and HiMgO@ZnMs groups. The rectangular boxes in the low-magnification images indicate the regions shown at higher magnification and used for ALP and OCN evaluation. Brown color represents positive osteogenic protein signals. Scale bar: 100 μ m and 40 μ m. (C, D) Quantitative percentage of ALP-positive (C) and OCN-positive (D) staining area. Data are mean $\pm$ SD ($n = 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

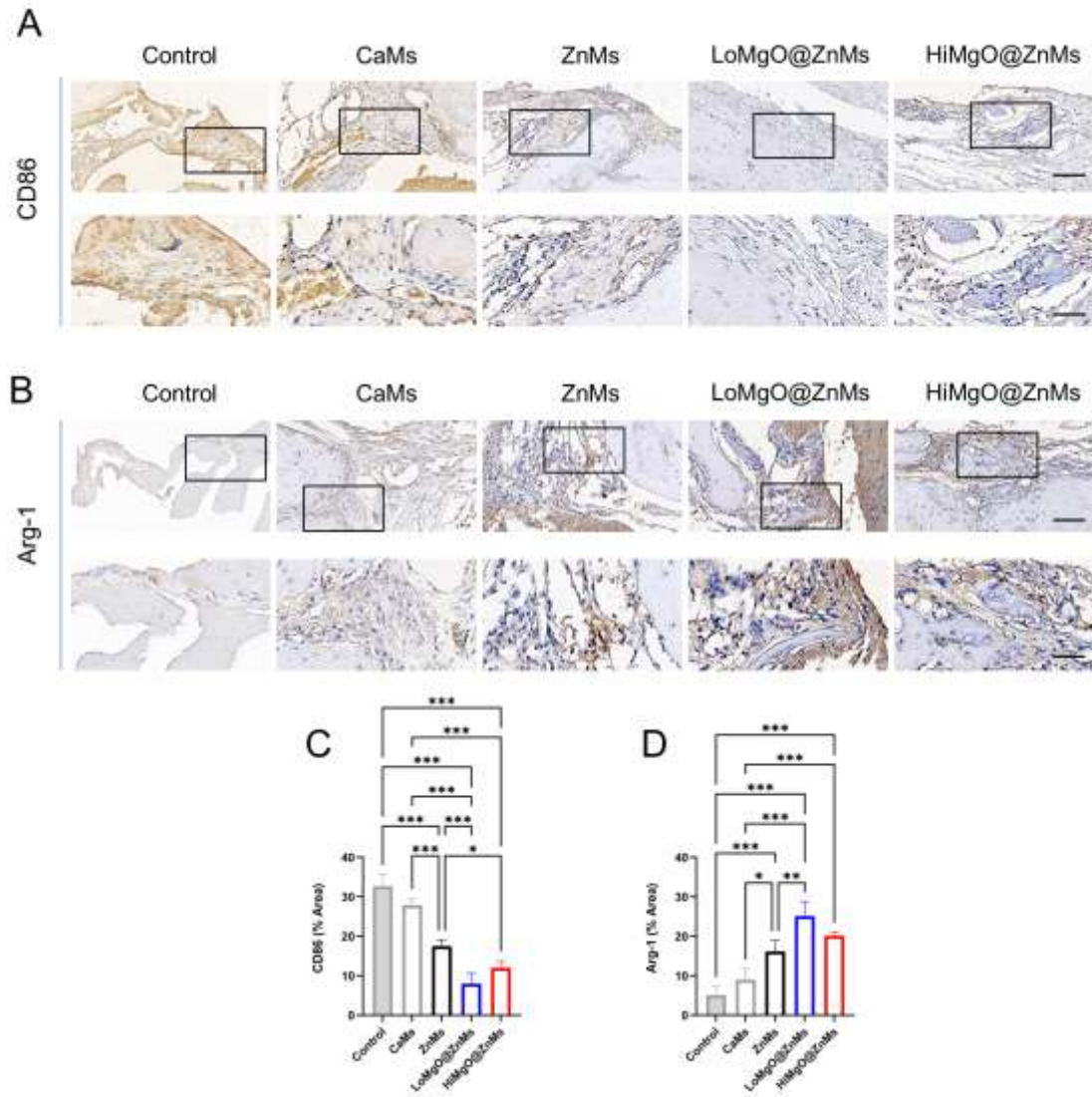


Figure 8. Immunohistochemical detection of macrophage-associated CD86 and Arg-1 expression at 8 weeks. (A, B) Panoramic and magnified images of CD86 (A) and Arg-1 (B) in the Control, CaMs, ZnMs, LoMgO@ZnMs, and HiMgO@ZnMs groups. The rectangular boxes in the low-magnification images indicate the regions shown at higher magnification and used for CD86 and Arg-1 evaluation. Brown staining indicates positive immune-marker signals. Scale bars: 100 and 40 μ m. (C, D) Percentages of CD86-positive (C) and Arg-1-positive (D) areas. Data are presented as mean $\pm$ SD ($n = 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

4.1. Role of the core-shell hydrogel architecture in ion release

Controlled release is important when bioactive ions are delivered from hydrogel systems for bone regeneration [12]. In the present study, a CS-rich shell was assembled on a ZnSA hydrogel core to

form an interfacial PEC layer. Ionically crosslinked alginate can undergo water uptake and crosslinker exchange in physiological media, leading to network relaxation and early cargo release [13,14]. The lower swelling and slower early mass loss of the CS-coated microspheres indicate that the shell stabilized the hydrogel network. Electrostatic interactions between protonated CS amino groups and alginate carboxyl groups provide a plausible basis for the observed barrier effect [15]. Consistent with this interpretation, CS-coated formulations released Zn^{2+} more gradually than bare ZnSA, and the MgO-loaded formulations continued to release Mg^{2+} through day 21. Because local tissue ion concentrations were not measured, the present data support moderated release kinetics but do not define an *in vivo* therapeutic concentration window.

4.2. Biological contributions of the material components

The individual material components provide complementary functions. Zn^{2+} is a catalytic cofactor for ALP and has been linked to Runx2-related osteogenic signaling [16,17]. Previous studies have also associated zinc-containing materials with regulation of inflammatory signaling and macrophage marker expression [18]. MgO serves as a magnesium reservoir; released Mg^{2+} has been reported to influence osteogenic and immune responses [19]. Although MgO can affect local pH, buffering was not directly measured in this study and is therefore considered a possible contributing mechanism rather than a demonstrated effect. The CS shell additionally supplies a cell-interactive surface [20,21]. Alginate–chitosan polyelectrolyte complexation can further stabilize hydrogel networks and regulate diffusion-controlled release [22]. The improved BMSC spreading observed on CS-coated formulations is consistent with the contribution of surface chemistry and topography to cell attachment.

4.3. Dose-dependent immunomodulatory responses to combined Mg and Zn exposure

Bioactive hydrogels can influence macrophage-associated immune responses through their surface chemistry, mechanical properties, and released biochemical cues [23]. LoMgO@ZnMs produced the most pronounced pro-regenerative marker profile in RAW264.7 cells, including higher *Arg-1*, *Il4*, and *Il10* and lower *iNOS*, *Il6*, and *Tnfa*. The weaker response at the higher MgO loading indicates that maximizing ion content did not maximize biological activity. This non-monotonic pattern may reflect concentration-dependent cellular responses to released Mg^{2+} and Zn^{2+} , although the present design does not isolate all main effects and interactions. In particular, an MgO-loaded Ca-crosslinked control was not included, so formal MgO-by-crosslinking-ion

interaction and synergism cannot be concluded. Furthermore, macrophages were not prestimulated with lipopolysaccharide; the data show modulation of basal marker expression rather than reversal of an established M1-like state.

4.4. Osteogenic responses to the composite hydrogel microspheres

Hydrogel microparticles have emerged as injectable, high-surface-area platforms for cell interaction and localized delivery of bioactive factors in bone regeneration [24]. The BMSC experiments independently showed that the composite hydrogel microspheres supported cell attachment and osteogenic differentiation. The CS-rich shell provided a textured, cell-interactive surface, while Zn^{2+} and Mg^{2+} supplied soluble biochemical cues. Zn^{2+} is involved in ALP activity and Runx2-associated osteogenic regulation [16,17,25,26], whereas Mg^{2+} has been linked to osteogenic signaling, including the MAPK/ERK pathway [27]. LoMgO@ZnMs consistently outperformed the higher MgO loading in ALP, mineralization, Runx2, and osteogenic-gene assays, indicating an optimal loading range rather than a simple dose-dependent increase. Because BMSCs and macrophages were tested in separate co-culture systems, the present results do not establish that macrophage-derived signals caused the osteogenic response.

4.5. *In vivo* bone regeneration and translational considerations

The *in vivo* data extend the material and cell findings to a rat calvarial defect model. LoMgO@ZnMs produced the highest bone-volume fraction, thicker trabeculae, broad collagen-associated matrix staining, and the strongest ALP and OCN staining after 8 weeks. The accompanying reduction in CD86 and increase in Arg-1 are consistent with a more pro-regenerative local immune environment. These outcomes support the use of the microspheres as an injectable filler for non-load-bearing defects. The limited mechanical robustness of many injectable hydrogels remains an important barrier to their application in load-bearing bone defects [28]. Translation will require additional evaluation of sterilization, batch reproducibility, long-term biosafety, ion exposure *in vivo*, and performance in clinically relevant pathological models.

4.6. Relevance to bioprinting and future bioink development

The present microspheres have several material-level features relevant to future incorporation into printed constructs, including a relatively narrow diameter range centered around 180–200 μm , stability in aqueous culture conditions, sustained $\text{Mg}^{2+}/\text{Zn}^{2+}$ release, and biological activity in cell and calvarial-defect models. These findings support their evaluation as a functional particulate

phase or modular building block in a printable carrier matrix. Microspheres can serve as modular components of bioprinted constructs by functioning as cell carriers, porogens, or localized depots for therapeutic molecules and ions. Recent studies have used interconnected cell microspheres to assemble vascularized, high-cell-density printed tissues ^[29], incorporated dexamethasone-loaded porous microspheres into a printable bioink for cartilage regeneration ^[30], and combined sustained-release microspheres with printed hydrogel scaffolds ^[31]. Related 3D-printed Zn/Mg-containing composite scaffolds demonstrate that controlled delivery of osteogenic ions can be integrated with mechanically supportive architectures ^[32]. Porous GelMA microspheres have also been used for multicellular assembly to promote coupled osteogenesis and angiogenesis ^[33], while magnesium-phosphate-containing printed scaffolds functionalized with antimicrobial peptides illustrate a complementary strategy for combining structural support with biological functionality ^[34].

The present work did not formulate these MgO@ZnSA/CS microspheres as a bioink or evaluate rheology, extrusion behavior, shape fidelity, interlayer stability, or post-printing cell viability. The current findings therefore establish sustained ion release and biological activity of the microspheres, but not printability. A next-stage bioprinting study should determine the microsphere loading fraction in a printable carrier matrix; characterize shear-thinning behavior, yield stress, storage and loss moduli, recovery after shear, and filament collapse; quantify pore and strand fidelity in multilayer constructs; and evaluate whether printing preserves ion-release kinetics and cell compatibility. Mechanically reinforced or hybrid printed scaffolds may be particularly appropriate because the hydrogel microspheres alone are not intended to bear physiological loads.

4.7. Limitations

This study has several limitations. First, intracellular signaling mechanisms underlying the observed macrophage and osteogenic responses were not directly tested. Second, RAW264.7 cells were evaluated without inflammatory prestimulation; consequently, the data describe basal phenotype-associated marker changes rather than M1-to-M2 repolarization. Third, the healthy rat calvarial model does not reproduce the altered inflammatory and metabolic environments associated with osteoporosis, diabetes, or infection. Fourth, no bioink formulation or printing experiment was performed, and the rheological and printability requirements for incorporating the microspheres into a 3D-bioprinted construct remain to be established. Finally, prior to clinical translation, long-term biosafety studies and large-animal validation should be conducted, and

consideration should be given to integrating the microspheres into mechanically supportive printed scaffolds.

5. Conclusion

Core-shell MgO@ZnSA/CS hydrogel microspheres were fabricated by ionotropic gelation and electrostatic deposition. The CS-rich PEC layer reduced swelling, improved stability in PBS, and extended Mg²⁺ and Zn²⁺ release. Among the formulations tested, LoMgO@ZnMs produced the strongest osteogenic response in BMSCs and the most pro-regenerative marker profile in RAW264.7 macrophages under basal culture conditions. It also generated the greatest bone regeneration in rat critical-sized calvarial defects after 8 weeks. These results support chitosan-coated, MgO-loaded zinc-alginate hydrogel microspheres as a tunable ion-delivery platform for non-load-bearing bone repair. Future work should define the mechanisms and *in vivo* safety of combined Mg²⁺/Zn²⁺ delivery, and integrate the microspheres into mechanically reinforced, printable carrier matrices. The following investigations will focus on systematically evaluating the rheological behavior, extrusion and print fidelity, multilayer construct stability, preservation of ion-release profiles, and post-printing cellular responses of microsphere-containing bioinks for bone bioprinting.

Acknowledgments

None.

Funding

This work was supported by the Liaoning Provincial Science and Technology Plan Joint Program-Natural Science Foundation-Doctoral Research Start-up Project (Grant No. 2024-BSLH-018) and General Project (Grant No. 2025-MSLH-033). This work was also supported by the Clinical Collaborative Project of Integrated Traditional Chinese and Western Medicine for Major Difficult and Complicated Diseases (Grant No. ZDYN-2024-A-137).

Conflict of interest

The authors declare no competing interests.

Author contributions

Conceptualization: Fang Cao, Dewei Zhao

Data curation: Fang Cao, Guoshuang Zheng, Junlei Li, Guangxiao Yin

Formal analysis: Fang Cao

Funding acquisition: Fang Cao

Investigation: Fang Cao, Guoshuang Zheng, Junlei Li, Zhihua Cheng, Guangxiao Yin, Liqun Song, Lu Li

Methodology: Fang Cao, Guoshuang Zheng, Junlei Li, Zhihua Cheng, Guangxiao Yin, Liqun Song, Lu Li

Project administration: Fang Cao, Jiawei Ying, Ke Yang, Dewei Zhao

Resources: Dewei Zhao

Software: Guoshuang Zheng, Liqun Song

Supervision: Fang Cao, Dewei Zhao

Validation: Fang Cao, Guoshuang Zheng, Guangxiao Yin

Visualization: Zhihua Cheng

Writing—original draft: Fang Cao

Writing—review & editing: Fang Cao, Guoshuang Zheng, Junlei Li, Zhijie Ma

Ethics approval and consent to participate

The animal study protocol was approved by the Laboratory Animal Welfare and Ethics Committee of Dalian University Affiliated Zhongshan Hospital (protocol code DW2025-028-01; approval date: July 2025).

Consent for publication

Not applicable.

Availability of data

The data presented in this study are available from the corresponding author upon reasonable request.

References

- [1] Zhang T, Wei Q, Zhou H, et al. Three-dimensional-printed individualized porous implants: A new "implant-bone" interface fusion concept for large bone defect treatment. *Bioact Mater.* 2021;6(11):3659-3670. doi:10.1016/j.bioactmat.2021.03.030
- [2] Miron RJ, Böhner M, Zhang Y, Bosshardt DD. Osteoinduction and osteoimmunology: Emerging concepts. *Periodontol 2000.* 2024;94(1):9-26. doi:10.1111/prd.12519

- [3] Chen R, Hao Z, Wang Y, et al. Mesenchymal Stem Cell-Immune Cell Interaction and Related Modulations for Bone Tissue Engineering. *Stem Cells Int.* 2022;2022:7153584. doi:10.1155/2022/7153584
- [4] Tomić SL, Babić Radić MM, Vuković JS, Filipović VV, Nikodinovic-Runic J, Vukomanović M. Alginate-based hydrogels and scaffolds for biomedical applications. *Mar Drugs.* 2023;21(3):177. doi:10.3390/md21030177
- [5] Song K, Yan X, Li S, et al. Preparation and detection of calcium alginate/bone powder hybrid microbeads for in vitro culture of ADSCs. *J Microencapsul.* 2015;32(8):811-819. doi:10.3109/02652048.2015.1094533
- [6] Liu Y, Liu S, Du J, et al. Mechanism and regulatory strategy study on promoting vascularized bone regeneration via intracellular zinc ion transport. *Bioact Mater.* 2025;53:875-892. doi:10.1016/j.bioactmat.2025.07.020
- [7] Yang B, Yao H, Yang J, Chen C, Shi J. Construction of a two-dimensional artificial antioxidant for nanocatalytic rheumatoid arthritis treatment. *Nat Commun.* 2022;13(1):1988. doi:10.1038/s41467-022-29735-1
- [8] Molenda M, Kolmas J. The Role of Zinc in Bone Tissue Health and Regeneration—a Review. *Biol Trace Elem Res.* 2023;201(12):5640-5651. doi:10.1007/s12011-023-03631-1
- [9] Goh CH, Heng PWS, Chan LW. Alginates as a useful natural polymer for microencapsulation and therapeutic applications. *Carbohydr Polym.* 2012;88(1):1-12. doi:10.1016/j.carbpol.2011.11.012
- [10] Nie X, Shi Y, Wang L, Abudurehman W, Yang J, Lin C. Study on the mechanism of magnesium calcium alloys/mineralized collagen composites mediating macrophage polarization to promote bone repair. *Heliyon.* 2024;10(9):e30279. doi:10.1016/j.heliyon.2024.e30279
- [11] Zhou H, He Z, Cao Y, et al. An injectable magnesium-loaded hydrogel releases hydrogen to promote osteoporotic bone repair via ROS scavenging and immunomodulation. *Theranostics.* 2024;14(9):3739-3759. doi:10.7150/thno.97412
- [12] Babu S, Shanmugavadivu A, Lekhavadhani S, Selvamurugan N. Magnesium-based composite scaffolds for therapeutic delivery in bone tissue engineering: Current advances and emerging frontiers. *J Magnes Alloys.* 2026;18:102029. doi:10.1016/j.jma.2026.102029

- [13] Liang X, Chen S, Liang Y, et al. Alginate-Based Hydrogels: Recent Progress in Preparation, Property Tuning, and Multifunctional Applications. *Gels*. 2026;12(2):182. doi:10.3390/gels12020182
- [14] Sangeetha K, Girija EK. Tailor made alginate hydrogel for local infection prophylaxis in orthopedic applications. *Mater Sci Eng C Mater Biol Appl*. 2017;78:1046-1053. doi:10.1016/j.msec.2017.04.154
- [15] Atma Y, Sadeghpour A, Murray BS, Goycoolea FM. Chitosan-alginate polyelectrolyte complexes for encapsulation of low molecular weight fish bioactive peptides. *Food Hydrocoll*. 2025;160:110789. doi:10.1016/j.foodhyd.2024.110789
- [16] Fernández JM, Molinuevo MS, McCarthy AD, Cortizo AM. Strontium ranelate stimulates the activity of bone-specific alkaline phosphatase: interaction with Zn^{2+} and Mg^{2+} . *Biometals*. 2014;27(3):601-607. doi:10.1007/s10534-014-9733-8
- [17] Park KH, Choi Y, Yoon DS, Lee KM, Kim D, Lee JW. Zinc Promotes Osteoblast Differentiation in Human Mesenchymal Stem Cells Via Activation of the cAMP-PKA-CREB Signaling Pathway. *Stem Cells Dev*. 2018;27(16):1125-1135. doi:10.1089/scd.2018.0023
- [18] Pan X, Ou M, Lu Y, Nie Q, Dai X, Liu O. Immunomodulatory zinc-based materials for tissue regeneration. *Biomater Adv*. 2023;152:213503. doi:10.1016/j.bioadv.2023.213503
- [19] Chen Y, Sheng W, Lin J, et al. Magnesium Oxide Nanoparticle Coordinated Phosphate-Functionalized Chitosan Injectable Hydrogel for Osteogenesis and Angiogenesis in Bone Regeneration. *ACS Appl Mater Interfaces*. 2022;14(6):7592-7608. doi:10.1021/acsami.1c21260
- [20] Leedy MR, Martin HJ, Norowski PA, Jennings JA, Haggard WO, Bumgardner JD. Use of chitosan as a bioactive implant coating for bone-implant applications. In: Jayakumar R, Prabakaran M, Muzzarelli RAA, eds. *Chitosan Biomaterials II*. Springer; 2011:129-165. doi:10.1007/12_2011_115
- [21] López-Valverde N, López-Valverde A, Cortés MP, Rodríguez C, Macedo De Sousa B, Aragonese JM. Bone Quantification Around Chitosan-Coated Titanium Dental Implants: A Preliminary Study by Micro-CT Analysis in Jaw of a Canine Model. *Front Bioeng Biotechnol*. 2022;10:858786. doi:10.3389/fbioe.2022.858786

- [22] Liu M, Zhu J, Song X, Wen Y, Li J. Smart Hydrogel Formed by Alginate-g-Poly(N-isopropylacrylamide) and Chitosan through Polyelectrolyte Complexation and Its Controlled Release Properties. *Gels*. 2022;8(7):441. doi:10.3390/gels8070441
- [23] Semeraro F, Herrera Millar VR, Aidos L, et al. Beyond Biomaterials: Engineering Bioactive Hydrogels as Immuno-Mechanobiological Niches for Osteochondral Regeneration. *Gels*. 2025;11(8):658. doi:10.3390/gels11080658
- [24] Bektas C, Mao Y. Hydrogel Microparticles for Bone Regeneration. *Gels*. 2024;10(1):28. doi:10.3390/gels10010028
- [25] Xin Y, Zhao N, Wang Y. Multiple roles of Runt-related transcription factor-2 in tooth eruption: bone formation and resorption. *Arch Oral Biol*. 2022;141:105484. doi:10.1016/j.archoralbio.2022.105484
- [26] Fu X, Li Y, Huang T, et al. Runx2/Osterix and Zinc Uptake Synergize to Orchestrate Osteogenic Differentiation and Citrate Containing Bone Apatite Formation. *Adv Sci (Weinh)*. 2018;5(4):1700755. doi:10.1002/advs.201700755
- [27] Qi H, Liu Y, Wu L, et al. MicroRNA-16, via FGF2 Regulation of the ERK/MAPK Pathway, Is Involved in the Magnesium-Promoted Osteogenic Differentiation of Mesenchymal Stem Cells. *Oxid Med Cell Longev*. 2020;2020:1-14. doi:10.1155/2020/3894926
- [28] Kaur K, Murphy CM. Advances in the Development of Nano-Engineered Mechanically Robust Hydrogels for Minimally Invasive Treatment of Bone Defects. *Gels*. 2023;9(10):809. doi:10.3390/gels9100809
- [29] Wang X, Zhang X, Gu L, et al. Porous-interconnected cell microspheres-based 3D bioprinting of vascularized high-cell-density tissues with channel-pore networks. *Chem Eng J*. 2026;536:176083. doi:10.1016/j.cej.2026.176083
- [30] Chen Z, Feng Y, Wang Y, et al. Dexamethasone-loaded PLGA porous microspheres used as 3D-bioprinted bioink promote tissue-engineered cartilage regeneration. *Int J Bioprint*. 2024;10(5):3396. doi:10.36922/ijb.3396
- [31] Zhang G, Song E, Yu P, et al. Three-dimensional-printed hydrogel scaffolds with neuregulin-1 sustained-release microspheres for enhanced dedifferentiation and myelin regeneration in peripheral nerve injury. *Int J Bioprint*. 2025;11(5):154–177. doi:10.36922/IJB025170168

- [32] Qiang L, Huang H, Shan J, et al. 3D-printed zinc/magnesium-doped hydroxyapatite-polycaprolactone composite scaffolds for angiogenesis and osteogenesis. *Int J Bioprint.* 2024;10(6):4243. doi:10.36922/ijb.4243
- [33] Hu X, Hu Q, Liu S, Zhang H. Synergy of engineered gelatin methacrylate-based porous microspheres and multicellular assembly to promote osteogenesis and angiogenesis in bone tissue reconstruction. *Int J Biol Macromol.* 2024;283(Pt 1):137228. doi:10.1016/j.ijbiomac.2024.137228
- [34] Zheng L, Li M, Liang C, Zu W, Zhao Y. Development of a 3D-printed polycaprolactone/magnesium phosphate composite scaffold functionalized with novel antimicrobial peptides for enhanced bone defect regeneration. *Int J Bioprint.* 2025;11(3):277–291. doi:10.36922/IJB025080060