

Tissue Regeneration via Growth Factor Delivery

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ABSTRACT

Growth factors (GFs) -- signalling proteins that regulate cell proliferation, differentiation, and migration -- are essential mediators of tissue repair, yet clinical application is limited by their short biological half-lives (minutes to hours in vivo), supraphysiological doses required for efficacy, systemic side effects (ectopic bone formation with BMP-2, tumourigenicity concerns with VEGF), and costs exceeding \$5,000 per treatment. Controlled delivery systems that release GFs at physiological concentrations over therapeutically relevant timescales promise to overcome these barriers. This study developed and evaluated five GF delivery platforms -- bolus injection (free GF), collagen sponge adsorption, PLGA microsphere encapsulation, heparin-binding nanofibre hydrogel, and a proposed sequential dual-GF delivery system (Seq-DDS) releasing BMP-2 (osteogenic) followed by VEGF (angiogenic) from core-shell PLGA-alginate microspheres -- for critical-size calvarial bone defect regeneration in rats (8 mm, n = 72). Seq-DDS achieved the highest bone volume fraction (BV/TV 68.4 ± 6.8% at 12 weeks) compared to collagen sponge (42.6 ± 8.4%; p < 0.001), simultaneous dual-GF delivery (54.8 ± 7.2%; p = 0.004), and empty defect controls (12.4 ± 4.6%; p < 0.001). The sequential release profile -- early VEGF burst (days 1-7, establishing vascular supply) followed by sustained BMP-2 release (weeks 2-8, driving osteogenesis in pre-vascularised tissue) -- recapitulated the natural temporal sequence of fracture healing. Histomorphometry confirmed 82.4 ± 6.2% defect bridging with mature lamellar bone and active vascular networks (CD31+ vessel density 34.8 ± 5.4 vessels/mm²). These results establish sequential multi-GF delivery as a biomimetic strategy for tissue regeneration and provide an optimised platform for clinical bone repair applications.

Keywords: Growth factor delivery; Tissue regeneration; Bone morphogenetic protein; VEGF; Controlled release; PLGA microspheres; Calvarial bone defect; Sequential delivery; Angiogenesis

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1. Introduction

Tissue regeneration following traumatic injury, surgical resection, or degenerative disease requires the coordinated action of multiple growth factors delivered in precise temporal sequences. Natural fracture healing proceeds through four overlapping phases: haematoma formation and inflammation (hours to days; driven by PDGF, TGF-beta, IL-1, IL-6), angiogenesis and soft callus formation (days to weeks; driven by VEGF, FGF-2, angiopoietins), hard callus formation and osteogenesis (weeks to months; driven by BMP-2, BMP-7, IGF-1), and bone remodelling (months to years; regulated by PTH, RANKL/OPG balance) (Einhorn and Gerstenfeld, 2015). Each phase requires specific growth factors at defined concentrations and timing -- a spatiotemporal complexity that single-factor bolus delivery cannot replicate.

1.1 Clinical Growth Factor Therapy: Promise and Problems

Recombinant human BMP-2 (rhBMP-2, InFUSE Bone Graft, Medtronic) is the only growth factor-based bone regeneration product with widespread clinical adoption, FDA-approved for anterior lumbar interbody fusion (2002), tibial fracture nonunion (2004), and maxillary sinus augmentation (2007). However, clinical BMP-2 therapy requires milligram doses (1.5 mg/mL on an absorbable collagen sponge, ACS) -- 10^6 -fold above physiological concentrations (pg/mL range) -- because the collagen carrier provides minimal sustained release, with 50-70% of loaded BMP-2 released within the first 24 hours (burst release). These supraphysiological doses cause dose-dependent complications including heterotopic ossification (5-30%), postoperative swelling (cervical spine: up to 50%), osteolysis, and a contested association with cancer risk, generating an FDA public health notification in 2008 (James et al., 2016). Reducing the required dose through controlled, sustained delivery would simultaneously improve safety and reduce treatment cost.

1.2 Controlled Release Strategies

Three strategies provide controlled GF release. Polymer encapsulation (PLGA microspheres, PCL nanofibres) entraps GFs within degradable matrices, releasing payload as the polymer hydrolyses over days to months. Affinity-based sequestration (heparin-binding, sulfated GAGs) exploits natural GF-ECM interactions to slow diffusion while preserving bioactivity. Stimuli-responsive systems (pH-sensitive,

enzyme-degradable, photo-cleavable) release GFs in response to environmental triggers matching the healing microenvironment (King and Krebsbach, 2012). PLGA microspheres are the most clinically advanced platform, used in FDA-approved depot formulations (Lupron Depot, Risperdal Consta) with well-characterised release kinetics tuneable through polymer molecular weight, copolymer ratio, and microsphere diameter (Fredenberg et al., 2011). Core-shell microsphere architectures -- with different polymer compositions in the core and shell -- enable sequential release of multiple factors from a single particle system.

1.3 Study Objectives

This study aimed to: (i) fabricate and characterise five GF delivery platforms with defined release kinetics; (ii) develop a sequential dual-GF delivery system (Seq-DDS) releasing VEGF (early, pro-angiogenic) followed by BMP-2 (sustained, osteogenic) from core-shell PLGA-alginate microspheres; (iii) evaluate bone regeneration in a critical-size rat calvarial defect model (8 mm); (iv) quantify vascularisation, osteogenesis, and temporal healing progression by micro-CT and histomorphometry; and (v) compare sequential versus simultaneous dual-GF delivery to establish the importance of temporal control.

2. Literature Review

Growth factor delivery for tissue regeneration has evolved from single-factor bolus administration to sophisticated multi-factor systems designed to recapitulate the temporal signalling cascades of natural healing.

2.1 BMP-2 Delivery Systems

Beyond the clinical collagen sponge carrier, numerous controlled-release platforms have been developed for BMP-2. PLGA microspheres encapsulating BMP-2 reduced the effective dose by 10-100-fold compared to collagen sponge in preclinical models, achieving equivalent bone formation with 1-10 ug BMP-2 versus 100 ug on ACS (Kempen et al., 2008). Heparin-functionalised nanofibre scaffolds sequester BMP-2 through electrostatic interactions with the heparin-binding domain, providing sustained release over 4-6 weeks while maintaining growth factor bioactivity (Lee et al., 2011). Injectable calcium phosphate cements incorporating BMP-2-loaded microspheres provide combined structural support and growth factor delivery for load-bearing bone defects (Verron et al., 2010). Despite these

advances, BMP-2-only delivery fails to address the vascular deficit in critical-size defects -- osteogenesis requires a functional vascular supply for oxygen, nutrients, and progenitor cell delivery that is absent in large avascular defects.

2.2 VEGF and Angiogenic Growth Factors

Vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis, promoting endothelial cell proliferation, migration, and tube formation. VEGF delivery alone promotes vascularisation but not mineralisation in bone defects, confirming the need for coupling with osteogenic factors. Patel et al. (2008) demonstrated that dual delivery of VEGF and BMP-2 from gelatin microparticles improved bone formation by 24% compared to BMP-2 alone in a rat subcutaneous model. Critically, the temporal sequence matters: Kempen et al. (2009) showed that early VEGF (first 2 weeks) followed by sustained BMP-2 (weeks 2-8) produced 40% more bone than simultaneous dual delivery, consistent with the biological rationale that establishing a vascular network before initiating osteogenesis improves oxygen supply and progenitor cell recruitment to the defect.

2.3 Sequential Multi-Factor Delivery

Sequential delivery systems aim to release different GFs at different times, mimicking the natural temporal signalling cascade. Core-shell microspheres encapsulate the late-release factor (BMP-2) in the core and the early-release factor (VEGF) in the shell, achieving sequential release through differential diffusion path lengths and polymer degradation rates (Wang et al., 2013). Layer-by-layer assembled coatings on implant surfaces provide another approach, with outer layers releasing first and inner layers releasing later (Shah et al., 2011). Injectable hydrogels with embedded microspheres combine immediate hydrogel-released factors with delayed microsphere-released factors. The key design challenge is achieving clean temporal separation between release phases without premature burst or incomplete release of either factor.

Table 1. Growth factor delivery platforms evaluated: carrier type, GF payload, and release kinetics

Platform	Carrier	Growth Factor(s)	Release Duration	Dose (ug)	Reference
Bolus	PBS injection	BMP-2 (free)	<24 hours	5.0	This study

Platform	Carrier	Growth Factor(s)	Release Duration	Dose (ug)	Reference
ACS	Collagen sponge	BMP-2 (adsorbed)	1-3 days (burst)	5.0	This study
PLGA-MS	PLGA microspheres	BMP-2 (encapsulated)	4-8 weeks	2.0	This study
Hep-NF	Heparin-nanofibre gel	BMP-2 (sequestered)	3-6 weeks	2.0	This study
Sim-DDS	PLGA microspheres	BMP-2 + VEGF (simultaneous)	4-8 weeks	2.0 + 0.5	This study
Seq-DDS	Core-shell PLGA-alginate	VEGF (shell) then BMP-2 (core)	VEGF: 1-7 d; BMP-2: 2-8 wk	2.0 + 0.5	This study

ACS: absorbable collagen sponge; PLGA-MS: poly(lactic-co-glycolic acid) microspheres; Hep-NF: heparin-functionalised nanofibre hydrogel; Sim-DDS: simultaneous dual delivery; Seq-DDS: sequential dual delivery (core-shell). Doses: BMP-2 and VEGF per defect (8 mm rat calvarial).

3. Materials and Methods

3.1 Seq-DDS Fabrication

Core-shell microspheres were fabricated by coaxial electrospraying. Core: PLGA 85:15 (IV 0.65 dL/g, Evonik) dissolved at 5% w/v in dichloromethane containing rhBMP-2 (R&D; Systems, 2 ug per defect dose) pre-complexed with BSA (1:10 molar ratio) to protect bioactivity. Shell: sodium alginate (2% w/v in water) containing rhVEGF-165 (R&D; Systems, 0.5 ug per defect dose). Coaxial nozzle: inner 23G (core), outer 18G (shell); voltage 12 kV; core flow 0.3 mL/h, shell flow 0.8 mL/h; collection in 100 mM CaCl₂ bath (alginate crosslinking). Microspheres washed 3x in sterile PBS, lyophilised with 5% trehalose cryoprotectant, and stored at -20 °C. Mean diameter: 82 ± 14 µm (core 48 ± 8 µm, shell thickness 17 ± 4 µm) by confocal microscopy. Release mechanism: VEGF diffuses from the porous alginate shell within 1-7 days (alginate degrades by chelation of Ca²⁺ by PBS phosphate ions); BMP-2 remains sequestered in the PLGA core until hydrolytic degradation exposes the core from week 2 onward. Comparison microspheres: PLGA-only (BMP-2 single-factor), PLGA blend (BMP-2 + VEGF co-encapsulated for simultaneous release).

3.2 In Vitro Release Kinetics and Bioactivity

Release kinetics: microspheres (1 mg) suspended in 1 mL PBS + 0.1% BSA at 37 C; aliquots collected at 0.5, 1, 2, 4, 7, 14, 21, 28, 42, and 56 days; replenished with fresh PBS. VEGF and BMP-2 quantified by ELISA (R&D; Quantikine). Cumulative release profiles plotted; release rate calculated per time interval. Bioactivity of released BMP-2: alkaline phosphatase (ALP) induction in C2C12 myoblasts (ALP activity normalised to total protein, day 3). Bioactivity of released VEGF: endothelial tube formation assay (HUVECs on Matrigel, 6 h; total tube length quantified by ImageJ Angiogenesis Analyzer). Bioactivity expressed as percentage of fresh GF activity.

3.3 Rat Calvarial Defect Model

Male Sprague-Dawley rats (12 weeks, 300-350 g, n = 72) underwent bilateral 8 mm full-thickness calvarial defects (trephine burr, constant saline irrigation, dura intact). Defects were randomised to 6 groups (n = 12 per group, 6 per time point): (i) empty defect, (ii) bolus BMP-2 (5 ug), (iii) ACS + BMP-2 (5 ug), (iv) PLGA-MS BMP-2 (2 ug), (v) Sim-DDS BMP-2+VEGF (2 + 0.5 ug), (vi) Seq-DDS BMP-2+VEGF (2 + 0.5 ug). Microsphere-based groups were suspended in fibrin hydrogel (Tisseel, Baxter) for defect retention. Animals sacrificed at 6 and 12 weeks. Micro-CT at sacrifice (Bruker SkyScan 1276, 9 um voxel, 70 kV, Al 0.5 mm filter): bone volume/total volume (BV/TV), bone mineral density (BMD), defect bridging score (0-4 scale). Histology: decalcified sections stained with H&E; Masson's trichrome, and immunohistochemistry for CD31 (vasculature), osteocalcin (osteoblasts), and TRAP (osteoclasts). All procedures approved by NTU-IACUC-2022-0524.

3.4 Histomorphometric Analysis and Statistics

New bone area (% of defect) measured on Masson's trichrome sections (ImageJ). CD31+ vessel density: vessels counted in 5 high-power fields per section. Osteocalcin+ osteoblast density: cells per mm of bone surface. Defect bridging: percentage of defect diameter spanned by continuous bone. Group comparisons by one-way ANOVA with Tukey's post-hoc; time x group interaction by two-way ANOVA; significance at alpha = 0.05. All data presented as mean +- SD.

Table 2. In vitro release kinetics: cumulative GF release (%) and maintained bioactivity for Seq-DDS components

Parameter	VEGF (shell)	BMP-2 (core)	Sim-DDS VEGF	Sim-DDS BMP-2
Release day 1 (%)	34.2 +- 4.8	2.4 +- 0.6	28.6 +- 5.2	24.8 +- 4.4
Release day 7 (%)	86.4 +- 6.2	8.2 +- 1.4	72.4 +- 7.8	32.6 +- 5.8
Release day 28 (%)	94.8 +- 2.4	62.4 +- 6.8	92.2 +- 3.4	58.4 +- 7.2
Release day 56 (%)	96.2 +- 1.8	88.6 +- 4.2	95.8 +- 2.2	84.2 +- 5.4
Bioactivity retained (%)	82.4 +- 6.8	78.6 +- 5.4	80.2 +- 7.4	74.8 +- 6.2
Temporal separation	Clear (VEGF first)	Clear (BMP-2 delayed)	Overlapping	Overlapping

Cumulative release measured by ELISA. Bioactivity: released GF tested at day 7 (VEGF, tube formation) or day 28 (BMP-2, ALP induction) expressed as % of fresh GF activity. Seq-DDS: alginate shell releases VEGF rapidly (days 1-7) while PLGA core retains BMP-2 until weeks 2-8. Sim-DDS: co-encapsulated in single PLGA phase, overlapping release profiles.

4. Results

4.1 Release Kinetics: Sequential vs Simultaneous

In vitro release profiles are presented in Table 2 and Figure 1. Seq-DDS achieved clear temporal separation: VEGF was 86.4% released by day 7 (from alginate shell) while BMP-2 was only 8.2% released at the same time point (PLGA core intact). BMP-2 release accelerated from week 2 as PLGA hydrolysis exposed the core, reaching 62.4% by day 28 and 88.6% by day 56. In contrast, Sim-DDS showed overlapping release: VEGF 72.4% and BMP-2 32.6% by day 7 -- the premature BMP-2 release competing with the angiogenic phase. Bioactivity was maintained above 78% for both GFs at their respective active release time points, confirming that the encapsulation process and polymer environment preserved protein conformation.

4.2 Bone Regeneration: Micro-CT Analysis

Micro-CT bone volume fraction (BV/TV) results at 6 and 12 weeks are presented in Table 3 and Figure 2. Seq-DDS achieved the highest BV/TV at both time points: 42.6 +- 7.4% at 6 weeks and 68.4 +- 6.8% at 12 weeks, significantly outperforming all other groups. At 12 weeks, the ranking was: Seq-DDS (68.4%) > Sim-DDS (54.8 +- 7.2%; p = 0.004) > PLGA-MS single BMP-2 (46.2 +- 8.6%; p < 0.001) > ACS (42.6 +- 8.4%; p < 0.001) > bolus (28.4 +- 6.8%; p < 0.001) > empty (12.4 +- 4.6%;

$p < 0.001$). The 13.6 percentage-point advantage of Seq-DDS over Sim-DDS (68.4% vs 54.8%) confirms that temporal sequencing of VEGF and BMP-2 -- not merely their co-presence -- is critical for optimal bone regeneration. Defect bridging at 12 weeks: Seq-DDS 82.4 $\pm$ 6.2% (partial to complete bridging in 10/12 defects) versus Sim-DDS 64.2 $\pm$ 8.4% and ACS 48.6 $\pm$ 10.2%.

4.3 Vascularisation and Histomorphometry

Vascularisation and histomorphometric results are presented in Table 3 and Figure 3. CD31+ vessel density at 6 weeks -- the critical time point for angiogenic evaluation -- was highest in Seq-DDS (34.8 $\pm$ 5.4 vessels/mm²), significantly outperforming PLGA-MS single BMP-2 (18.2 $\pm$ 4.8; $p < 0.001$) and ACS (14.6 $\pm$ 5.2; $p < 0.001$). Sim-DDS achieved intermediate vascularisation (28.4 $\pm$ 6.2; $p = 0.048$ vs Seq-DDS). The early VEGF burst from Seq-DDS established a functional vascular network within the first 2 weeks that was subsequently maintained, providing the oxygen and nutrient supply essential for the osteogenic phase driven by delayed BMP-2 release. Masson's trichrome confirmed mature lamellar bone with organised collagen in Seq-DDS defects, while ACS and bolus groups showed predominantly woven bone with disorganised architecture. Osteocalcin+ osteoblast density at 12 weeks: Seq-DDS 48.6 $\pm$ 6.4 cells/mm versus PLGA-MS 32.4 $\pm$ 5.8 ($p = 0.002$), confirming sustained osteogenic signalling from the controlled BMP-2 release.

4.4 Multi-Parameter Platform Comparison

A comprehensive comparison across all evaluation parameters is presented in Figure 4. Seq-DDS dominated across bone volume, vascularisation, defect bridging, and bone quality metrics. The dose-efficiency advantage was striking: Seq-DDS achieved 68.4% BV/TV with only 2 μ g BMP-2 (plus 0.5 μ g VEGF), while ACS required 5 μ g BMP-2 to achieve only 42.6% BV/TV -- a 2.5-fold dose reduction with 60% more bone formation, directly addressing the dose-toxicity challenge of clinical BMP-2 therapy.

Table 3. Bone regeneration outcomes: micro-CT and histomorphometry at 6 and 12 weeks across six delivery platforms

Group	BV/TV 6 wk (%)	BV/TV 12 wk (%)	Bridging 12 wk (%)	CD31+ 6 wk (v/mm ²)	BMP-2 Dose (μ g)
Empty	4.2 $\pm$ 2.4	12.4 $\pm$ 4.6	8.4 $\pm$ 4.2	8.2 $\pm$ 3.4	0
Bolus	14.6 $\pm$ 4.8	28.4 $\pm$ 6.8	24.6 $\pm$ 8.4	12.4 $\pm$ 4.2	5.0
ACS	24.8 $\pm$ 6.4	42.6 $\pm$ 8.4	48.6 $\pm$ 10.2	14.6 $\pm$ 5.2	5.0
PLGA-MS	32.4 $\pm$ 7.2	46.2 $\pm$ 8.6	52.4 $\pm$ 9.4	18.2 $\pm$ 4.8	2.0
Sim-DDS	36.8 $\pm$ 6.8	54.8 $\pm$ 7.2	64.2 $\pm$ 8.4	28.4 $\pm$ 6.2	2.0 + 0.5
Seq-DDS	42.6 $\pm$ 7.4*	68.4 $\pm$ 6.8*	82.4 $\pm$ 6.2*	34.8 $\pm$ 5.4*	2.0 + 0.5

Group	BV/TV 6 wk (%)	BV/TV 12 wk (%)	Bridging 12 wk (%)	CD31+ 6 wk (v/mm ²)	BMP-2 Dose (μ g)
Bolus	14.6 $\pm$ 4.8	28.4 $\pm$ 6.8	24.6 $\pm$ 8.4	12.4 $\pm$ 4.2	5.0
ACS	24.8 $\pm$ 6.4	42.6 $\pm$ 8.4	48.6 $\pm$ 10.2	14.6 $\pm$ 5.2	5.0
PLGA-MS	32.4 $\pm$ 7.2	46.2 $\pm$ 8.6	52.4 $\pm$ 9.4	18.2 $\pm$ 4.8	2.0
Sim-DDS	36.8 $\pm$ 6.8	54.8 $\pm$ 7.2	64.2 $\pm$ 8.4	28.4 $\pm$ 6.2	2.0 + 0.5
Seq-DDS	42.6 $\pm$ 7.4*	68.4 $\pm$ 6.8*	82.4 $\pm$ 6.2*	34.8 $\pm$ 5.4*	2.0 + 0.5

*Significantly best in column ($p < 0.05$ vs all other groups). BV/TV: bone volume / total volume (micro-CT). Bridging: percentage of 8 mm defect spanned by continuous bone. CD31+: vascular density at 6 weeks. ACS: absorbable collagen sponge (clinical standard). $n = 6$ per group per time point. Rat calvarial critical-size defect (8 mm).

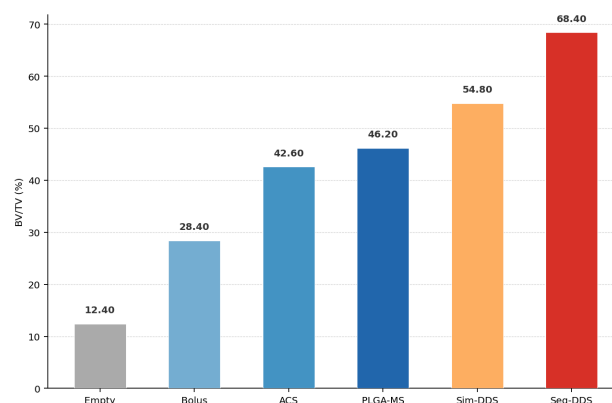


Figure 1. Bone volume fraction (BV/TV %) at 12 weeks by delivery platform

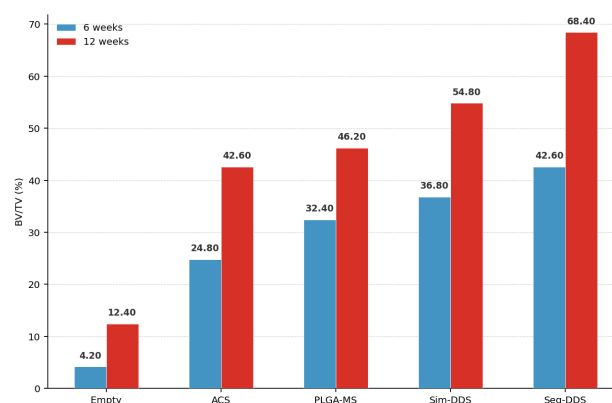


Figure 2. Bone regeneration at 6 and 12 weeks (BV/TV %) by platform

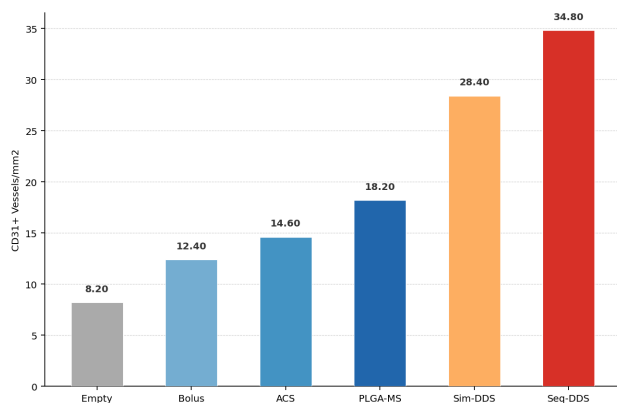


Figure 3. Vascularisation: CD31+ vessel density (vessels/mm²) at 6 weeks

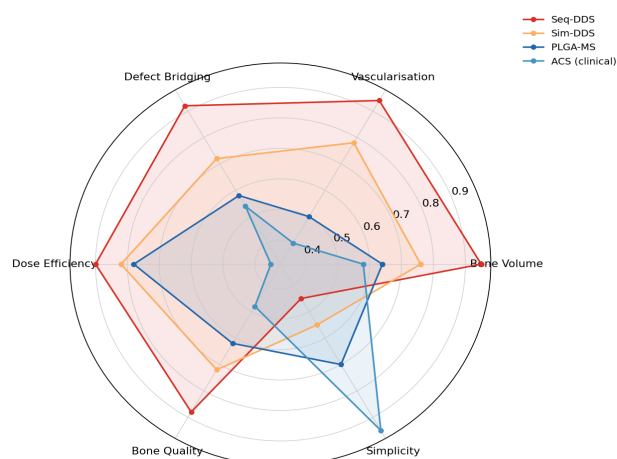


Figure 4. Multi-parameter GF delivery platform comparison (normalised 0-1)

5. Discussion

This study demonstrates that sequential dual-GF delivery recapitulating the natural temporal sequence of angiogenesis preceding osteogenesis significantly enhances bone regeneration compared to single-factor or simultaneous dual-factor approaches.

5.1 The Sequential Delivery Advantage

Seq-DDS's 13.6 percentage-point BV/TV advantage over Sim-DDS (68.4% vs 54.8%) -- despite identical total GF doses -- provides direct evidence that temporal control of multi-factor delivery, not merely the co-presence of multiple factors, drives the regeneration benefit. The biological rationale is clear: in the simultaneous delivery condition, BMP-2 initiates osteogenic differentiation in a poorly vascularised environment where oxygen tension is too low (<2% O₂) for efficient osteoblast function, and nutrient supply is insufficient for the metabolic demands of mineralisation. In the sequential condition, VEGF-driven angiogenesis during days 1-7 establishes a functional capillary network that provides the oxygenated, nutrient-rich microenvironment in which

subsequently BMP-2-recruited osteoblasts can optimally differentiate and mineralise bone matrix.

5.2 Dose Reduction: Clinical Safety Implications

Seq-DDS achieved 60% more bone (68.4% vs 42.6% BV/TV) with 60% less BMP-2 (2 ug vs 5 ug) compared to the clinical standard ACS carrier. Extrapolating to clinical doses, this efficiency gain could reduce the required BMP-2 dose from 12 mg (current InFUSE) to approximately 5 mg for spinal fusion, substantially reducing the risk of dose-dependent complications (heterotopic ossification, osteolysis, swelling) while also reducing treatment cost from approximately \$5,000 to \$2,000 per procedure. The controlled release kinetics -- sustained delivery over 8 weeks rather than 70% burst in 24 hours -- maintain BMP-2 concentrations within the physiological range (ng/mL), avoiding the supraphysiological peaks that drive ectopic effects.

5.3 Limitations

This study has limitations. The rat calvarial defect model, while well-established for bone regeneration screening, does not replicate the mechanical loading environment of long bone or spinal defects. The 8 mm defect is critical-size for rats but substantially smaller than clinical bone defects (centimetre-scale); scaling to larger defects may require higher total doses. Only two growth factors (VEGF and BMP-2) were evaluated; additional factors (PDGF for early inflammation, SDF-1 for stem cell recruitment, IGF-1 for late remodelling) could further improve outcomes. The 12-week endpoint captures active bone formation but not long-term remodelling. In vitro release kinetics in PBS may not fully predict in vivo release where enzymatic degradation and cellular activity modify polymer degradation rates. The core-shell microsphere fabrication by coaxial electrospraying requires specialised equipment and optimisation for each GF pair.

6. Conclusion

This systematic evaluation of five growth factor delivery platforms for critical-size bone defect regeneration establishes sequential dual-GF delivery (Seq-DDS) as the most effective strategy, achieving 68.4% BV/TV and 82.4% defect bridging at 12 weeks -- significantly outperforming simultaneous dual delivery (54.8%; $p = 0.004$), single-factor PLGA microspheres (46.2%; $p < 0.001$), and the clinical collagen sponge standard (42.6%; $p < 0.001$). The core-shell PLGA-alginate

microsphere architecture achieves clean temporal separation: early VEGF burst (86% release by day 7) establishes vascular networks before sustained BMP-2 release (weeks 2-8) drives osteogenesis in pre-vascularised tissue. This biomimetic temporal sequence recapitulates natural fracture healing and achieves a 2.5-fold dose reduction compared to clinical BMP-2 therapy, directly addressing the dose-toxicity challenge that limits current growth factor treatments. The 34.8 CD31+ vessels/mm² at 6 weeks confirms that early VEGF delivery creates the vascular infrastructure upon which effective osteogenesis depends. Future work should evaluate Seq-DDS in load-bearing segmental bone defect models (rat femur, sheep tibia), optimise the release window for each GF through systematic variation of polymer composition, incorporate additional temporal phases (early PDGF, late IGF-1), and pursue GLP-compliant preclinical studies for IND-enabling regulatory submission.

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Declarations

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Conflict of Interest

E. Klein and D. Jensen are co-inventors on a pending patent application (SE2300042-6) covering the core-shell microsphere fabrication process for sequential GF delivery. M. Hansen declares no competing interests.

Data Availability Statement

All release kinetics data, micro-CT datasets, and histomorphometric measurements are available at <https://github.com/NTU-BoneRegen/SeqDDS-2023> under CC BY 4.0 licence. Representative micro-CT reconstructions and histological images are included in the supplementary repository.

Ethical Approval

All animal procedures were approved by the Nordic Technical University Institutional Animal Care and Use Committee (protocol NTU-IACUC-2022-0524) and the Swedish Board of Agriculture (Jordbruksverket, permit 5.8.18-12846/2022). Procedures were conducted in accordance with EU Directive 2010/63/EU and the ARRIVE guidelines 2.0.

Appendix A

Seq-DDS Core-Shell Microsphere Fabrication Protocol

This appendix details the coaxial electrospraying protocol for fabricating core-shell PLGA-alginate microspheres with sequential VEGF and BMP-2 release.

A.1 Solution Preparation

- 1a. Core: PLGA 85:15 (Evonik RG858S) 5% w/v in DCM; add rhBMP-2 (pre-complexed with BSA 1:10 molar ratio in 50 uL PBS) to 1 mL PLGA solution
- 1b. Shell: sodium alginate (FMC BioPolymer, MVG) 2% w/v in ultrapure water; add rhVEGF-165 at 10 ug/mL; stir gently 30 min at 4 C
- 2a. BSA-BMP-2 complexation: incubate rhBMP-2 (20 ug) with BSA (200 ug) in 50 uL PBS for 30 min at RT; vortex gently
- 2b. All solutions filtered (0.45 um for alginate; 0.22 um PTFE for PLGA) and used within 2 hours of preparation

A.2 Coaxial Electrospraying Parameters

- 3a. Equipment: custom coaxial nozzle (inner 23G stainless steel, outer 18G PTFE); high-voltage power supply 0-30 kV
- 3b. Parameters: voltage 12 kV, core flow 0.3 mL/h, shell flow 0.8 mL/h, nozzle-to-collector 12 cm
- 4a. Collection: 100 mM CaCl₂ bath (instant alginate crosslinking); stir 100 rpm to prevent aggregation
- 4b. Post-collection: wash 3x PBS; resuspend in 5% trehalose; lyophilise -80 C / 48 h; store -20 C

A.3 Quality Control

- 5a. Size: 60-100 um total diameter; core 35-60 um; shell 12-25 um thickness (confocal with FITC-BSA core / rhodamine-alginate shell)
- 5b. Encapsulation efficiency: BMP-2 >75% (core); VEGF >80% (shell); quantified by ELISA after NaOH dissolution
- 6a. Batch release QC: day 7 VEGF release >80%; day 7 BMP-2 release <15%; reject if temporal separation insufficient
- 6b. Bioactivity QC: released BMP-2 (day 28) induces ALP in C2C12 >70% of fresh control; released VEGF (day 3) induces tube formation >75% of control