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Low acyl gellan gum supports bone tissue development and regeneration

Marran Aldossari, Faris Aldossari and Dong-Shik Kim*

Department of Chemical Engineering, University of Toledo, Toledo, USA

* Correspondence author; E-mail: dong.kim@utoledo.edu.

Highlights:

- LA-GAGR and Mini-GAGR promoted osteogenic responses in MC3T3-E1 cells.
- Mini-GAGR showed greater expression of several genes compared with LA-GAGR.
- LA-GAGR and Mini-GAGR promoted greater matrix mineralization compared to other polysaccharides.

Abstract: Advances in bone tissue engineering depend on biomaterials that can promote osteogenic differentiation and matrix mineralization for functional bone regeneration. Gellan gum (GG), a naturally derived polysaccharide, which is biocompatible, has tunable gelation, and has potential bioactivity, is considered an ideal candidate for composite scaffolds. This study evaluates the osteogenic potential of two GG variants, low acyl gellan gum (LA-GAGR) and Mini-GAGR, and compares their effects with other polysaccharides, hyaluronic acid (HA), welan gum (WG), and dextran. MC3T3-E1 mouse pre-osteoblast cells were cultured with each polysaccharide in both liquid and hydrogel formulations over 3, 7, and 14 days. Osteogenic responses were assessed through Alizarin Red Staining (ARS) for calcium deposition, alkaline phosphatase (ALP) staining, and gene expression of key osteogenic markers. LA-GAGR (500 kDa) and Mini-GAGR (760 Da) in their hydrogel form showed higher calcium deposition, and upregulation of osteogenesis-associated genes (RUNX2, SP7) and extracellular matrix (ECM)–associated genes (OCN, OPN, and COL1), when compared with other polysaccharides. Notably, Mini-GAGR showed higher effects than LA-GAGR on osteogenic markers, matrix-associated gene expression, while LA-GAGR, with its higher molecular weight, showed higher ALP staining intensity. Overall, these results suggest that LA-GAGR and Mini-GAGR exhibit osteogenic potential under the tested *in vitro* conditions, supporting their further investigation as polysaccharide-based materials for bone tissue engineering applications.

Keywords: gellan gum; bone tissue engineering; osteogenesis; ALP; ARS; bone regeneration

1. Introduction

Repairing bone defects arising from trauma, inflammation, tumors, osteoporosis, or severe fractures remain a major clinical challenge. In the United States, approximately 500,000–600,000 bone grafting procedures are done annually [1] and more than 2.2 million reported worldwide [2]. To address this

growing need, biomaterial-based strategies have gained increasing attention as promising alternatives to traditional grafts because they can support bone regeneration and overcome limitations such as donor site complications and limited graft availability [3]. The success of these approaches largely depends on their ability to replicate the physiological microenvironment that regulates osteogenesis, thereby promoting more effective and sustained bone healing [4]. Following bone injury, repair involves a complex signaling cascade of inflammatory, angiogenic, and osteogenic events [5,6]. During early inflammatory response, mesenchymal stem cells (MSCs) are recruited to the defect site, where they differentiate into osteoblasts and subsequently coordinate cell proliferation, extracellular matrix (ECM) formation, maturation, and mineralization [5]. As the matrix matures, osteoblasts become embedded and differentiate into osteocytes. Osteocytes are known to play a central role in maintaining bone homeostasis by controlling the activity of both osteoclasts and osteoblasts [6].

Recent efforts in bone tissue engineering have therefore focused on developing biofunctional scaffolds that actively modulate the immune, osteogenic and angiogenic microenvironment to promote healing, rather than serving solely as passive structural matrices [7]. A wide range of natural and synthetic biomaterials, such as collagen, hyaluronic acid, calcium phosphate ceramics, hydroxyapatite, and bioactive glass, have been investigated [8,9]. Each material offers distinct advantages but also presents inherent limitations in terms of biological activity, mechanical performance, degradation behavior, or clinical translation [8–10]. Naturally derived polymers have garnered increasing attention owing to their excellent biocompatibility and structural versatility. Their performance can be further enhanced through chemical modification, composite engineering, and fabrication strategies [8]. Polymers can form hydrogels which provide a highly hydrated, porous, and conformable three-dimensional microenvironment that promotes cell infiltration and angiogenesis, while reducing inflammatory responses following implantation [11,12].

Polysaccharides are among the most extensively investigated naturally derived polymers for bone tissue engineering, owing to properties such as hydrophilicity, gelation behavior, permeability, and tunable degradation [13,14]. They are also non-toxic, posing a lower risk of adverse reactions, and exhibit a wide range of biological activities, including antioxidants, antibacterial, anti-inflammatory, and antidiabetic properties [15,16]. These polymers have demonstrated to support cell adhesion, proliferation, and differentiation, and promote mineralization by mimicking the native ECM [17]. Structurally, polysaccharides may be linear or branched, homo- or heteropolymers, and their ability to form networks via electrostatic interactions and hydrogen bonding enhances scaffold integrity and functionality [18]. Studies have shown that many polysaccharides significantly boost osteogenic differentiation. For instance, combining hyaluronic acid (HA) with diacylglycerol enhances osteogenic differentiation of human amniotic MSCs, while dextran sulfate-enriched cultures exhibit improved ECM deposition [19,20]. Similarly, long-term culture of human osteoblasts (hOBs) on gellan gum–hydroxyapatite (GG-HAp) matrices has been shown to promote osteoblast-to-osteocyte transition [21]. These findings support the use of polysaccharides in bone tissue engineering as bioactive scaffolds that support osteogenic activities.

Gellan gum (GG), a naturally derived polysaccharide, has been investigated as a promising material in regenerative medicine and osteochondral tissue engineering because of its favorable physicochemical properties, mechanical stability, and biological compatibility [22,23]. GG is a linear, negatively charged, exopolysaccharide of ~500 kDa produced by *Sphingomonas* bacteria. It is composed

of repeating units of tetrasaccharide, containing two β-D-glucose residues, one β-D-glucuronic acid residue and one α-L-rhamnose residue [24]. The degradation rate of GG typically ranges from one to four weeks, making it well-suited for applications in the initial phases of tissue regeneration, also minimizing long-term scaffold interference [25]. Its gelation properties, mechanical stability, and responsiveness to external stimuli, make it an attractive candidate for scaffold fabrication [26]. A recent study by Liu *et al.* used an injectable GG-based hydrogel system, enhanced with nano-hydroxyapatite (nHAp) and magnesium sulfate ($MgSO_4$), aimed at promoting bone regeneration and angiogenesis [27] Their *in vitro* experiments demonstrated effective osteogenic differentiation of bone marrow MSCs and the formation of vascular-like structures by human umbilical vein endothelial cells (HUVECs).

The present study focuses on evaluating the osteogenic potential of two GG derivatives, low acyl gellan gum (LA-GAGR) and its low molecular weight fragment, Mini-GAGR. The reduced acyl content in LA-GAGR enables the formation of firm, brittle, translucent gels with high thermal stability and enhanced mechanical strength [28]. On the other hand, Mini-GAGR, a ~760 Da cleavage product of LA-GAGR that retains the same repeating tetrasaccharide unit, has been explored as a promising biomaterial. Its lower molecular weight may facilitate faster diffusion and penetration into the biological matrix [29]. This property, combined with its potential osteogenic activity as a LA-GAGR derivative, prompted its inclusion in this study to explore its role in bone cell regeneration.

The performance of both GG derivatives was compared with other polysaccharides, namely HA, welan gum (WG), and dextran. These materials were selected because they represent distinct structural and functional classes of natural polysaccharides that are commonly used in tissue engineering. HA has high biocompatibility and is widely used in scaffold systems, but it has limited ability to directly induce osteogenesis making it a good comparator for GG derivatives [30]. WG shares a similar backbone structure with GG, which enables comparison of side-chain effects on cell behavior. Dextran, on the other hand, serves as a neutral polysaccharide control with minimal bioactivity [31,32]. MC3T3-E1 mouse pre-osteoblast cells were cultured with each polysaccharide in liquid or hydrogel form and evaluated after 3, 7, and 14 days. This experimental design enabled us to compare the osteogenic responses to LA-GAGR and Mini-GAGR with other structurally different polysaccharides in different formulations, providing insight into the intrinsic osteogenic potential of the GG derivatives.

The osteogenic outcomes were assessed using alkaline phosphatase (ALP) staining and Alizarin Red Staining (ARS). ALP was used as an indicator of osteoblastic differentiation, and ARS detected calcium deposition and mineralization. Gene expression analysis was further carried out using quantitative real-time polymerase chain reaction (qRT-PCR) to assess changes in osteogenesis-associated gene transcription. The transcription factors RUNX2 and SP7 (Osterix, Osx), both central to osteoblast commitment [33], were examined alongside other markers of matrix maturation and mineralization. This included osteocalcin (OCN), a hallmark of mature osteoblast activity and bone mineralization; osteopontin (OPN), which regulates mineralization and mediates adhesion during tissue repair; and type I collagen (COL1), the structural backbone of the bone ECM [34]. In addition, SOX9, a key regulator of chondrogenic/osteochondroprogenitor specification, was analyzed because its downregulation is essential for commitment toward the osteogenic lineage [35]. Lastly, the vascular endothelial growth factor (VEGF) was included as a marker associated with pro-angiogenic signaling, given the fact that vascularization is indispensable for effective bone regeneration [36].

2. Methods

2.1. Materials

LA-GAGR (500 KDa) was purchased from CP-Kelco (San Diego, USA). Glacial acetic acid, sodium acetate, β-glucosidase, Dulbecco's Modified Eagle Medium (DMEM), trypsin, fetal bovine serum (FBS), phosphate-buffered saline (PBS), Alizarin Red S, and Alkaline Phosphatase staining kits were all obtained from Sigma-Aldrich (Milwaukee, USA). The MC3T3-E1 pre-osteoblast cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA). The PureLink™ RNA Mini Kit, High-Capacity cDNA Reverse Transcription Kit, and PowerUp™ SYBR™ Green Master Mix were purchased from Thermo Fisher Scientific (Waltham, USA). Gene-specific primers were purchased from Integrated DNA Technologies (IDT, Coralville, USA). RNA quantification was performed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA).

2.2. Enzymatic cleavage of LA-GAGR

The preparation of Mini-GAGR was carried out according to the method reported by Murphy *et al.* [29]. A buffer solution was prepared by mixing 0.1 M acetic acid with 0.1 M sodium acetate, adjusted to a pH of 5.0. An enzyme solution was created by dissolving β-glucosidase into a portion of this buffer solution to achieve a concentration of 0.1 mg/mL. An LA-GAGR solution was prepared by adding 0.6 g of LA-GAGR to 200 mL of the buffer solution, to obtain a concentration of 3 mg/mL. Two sample solutions were then prepared in separate 100 mL glass vials by combining 20 mL of the enzyme solution with 80 mL of the LA-GAGR solution. These samples were incubated at 37 °C with agitation at 150 rpm. After 72 hours, the second vial was removed from the incubator and immediately placed in a hot water bath for 5 minutes, followed by an ice-water bath for an additional 5 minutes to halt enzymatic hydrolysis. The resulting product was labeled as Mini-GAGR. The vial was then centrifuged at 10,000 g force to separate the enzyme and impurities, and the supernatant was carefully removed by pipetting. Excess liquid was eliminated from the sample using a vacuum oven set at −60 cmHg gauge and 40 °C until all liquid had evaporated.

2.3. Cell culture on liquid and hydrogel polysaccharide formulations

MC3T3-E1 pre-osteoblast cells were maintained in complete α-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 50 μg/mL ascorbic acid. Cells were cultured in T-75 flasks at 37 °C in a humidified incubator with 5% CO_2. The culture medium was refreshed every 2 days. For experimental treatments, cells were harvested at ~80% confluence and seeded into 24-well plates at a density of 2×10^5 cells per well.

Polysaccharide solutions were prepared at the specified concentrations (% w/v), including LA-GAGR (0.01%), Mini-GAGR (0.1%), HA (0.5%), dextran (0.9%), and WG (0.01%) for liquid phase, and LA-GAGR (1.0%), Mini-GAGR (3.0%), HA (1.0%), dextran (7.0%), and WG (1.0%) for hydrogel, and dispensed directly into each well prior to cell seeding.

For the liquid formulations, the respective polysaccharide solutions were dispensed into the wells, followed by the addition of MC3T3-E1 cells. For the gel-like formulations, 0.2 mL of each polysaccharide formulation was dispensed directly into each well and allowed to cool to room temperature, during which

gel-like behavior was observed. Following formation of the gel/gel-like formulations, MC3T3-E1 cells were seeded directly onto the surface. After cell seeding, the cultures were incubated at 37 °C in a humidified incubator with 5% CO_2, and the culture medium was replaced every 2 days.

The polysaccharide concentrations were selected based on their material-specific formulation and rheological behavior to obtain the intended liquid or gel/gel-like states under the experimental conditions. Because the tested polysaccharides differ in molecular structure, molecular weight, intermolecular interactions, and concentration-dependent rheological behavior, different concentrations were required to achieve the respective formulation states [37].

2.4. qRT-PCR analysis

To evaluate the expression of osteogenic and angiogenic marker genes (RUNX2, SP7, COL1, OPN, OCN, SOX9, and VEGF), MC3T3-E1 cells cultured with different polysaccharides were harvested on Days 3, 7, and 14. qRT-PCR was performed to assess the gene expression levels under different conditions. Total RNA was extracted using the PureLink™ RNA Mini Kit according to the manufacturer's instructions. RNA concentration and purity were evaluated using a NanoDrop™ spectrophotometer. First-strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol.

qRT-PCR was conducted using PowerUp™ SYBR™ Green Master Mix with gene-specific primers. The thermal cycling conditions followed the IDT recommended protocol: polymerase activation at 95 °C for 3 minutes, followed by 40 amplification cycles, each consisting of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. A final hold was set at 4 °C for up to 24 hours, if needed. The housekeeping genes β-actin and Gusb were used as the internal control for normalization, and relative gene expression levels were calculated using the 2^−ΔΔCt method. Primer sequences used in this study are listed in Table 1.

Table 1. Primer sequences of genes tested.

Gene	Sequences (5' → 3')	GI accession	Product length (bp)
RUNX2	For: CCGTCCACTGTCACTTTAATAGC Rev: GTAGCCAGGTTCAACGATCTG	NR_073425	134
SP7	For: CTTCTTTGTGCCTCCTTTCC Rev: GCGTCCTCTCTGCTTGA	NM_130458	135
COL1	For: CATTGTGTATGCAGCTGACTTC Rev: CGCAAAGAGTCTACATGTCTAGG	NM_007742	134
OPN	For: TCGTCATCATCGTCGTCCA Rev: AGAATGCTGTGTCCTCTGAAG	NM_001204201	100
OCN	For: GGCAGCGAGGTAGTGAAGAG Rev: TGGTAGTGAAGAGGTAGTGAAG	NM_001204201	100
SOX9	For: GTCTCTTCTCGCTCTCGTTC Rev: CGACCCATGAACGCCTT	NM_011448	139
VEGF	For: GACTTCTGCTCTCCTTCTGTC Rev: CCGAAACCATGAACTTTCTGC	NM_001025250	115

2.5. Alizarin red s staining

ARS staining was performed on Day 7 and 14 to assess calcium deposition as a measure of matrix mineralization [38]. Cells cultured with the polysaccharides in liquid and hydrogel formulations were

analyzed to compare mineralization under the two culture conditions. The cells were washed with 1.5 mL of Dulbecco's PBS (free of Ca^{2+}/Mg^{2+}) and then were fixed in 10% neutral buffered formalin for 15 minutes, followed by three washes with deionized water (5–10 minutes each). Subsequently, 1 mL of ARS staining solution was added, and cells were incubated in the dark at room temperature for 30 minutes. An excess stain was removed through four washes with deionized water. Calcium deposits were visualized as red staining under the microscope. ImageJ software was used to quantify the staining intensities.

2.6. Alkaline phosphatase staining

ALP is an enzyme expressed in bone-forming cells and is considered an early marker of osteoblast differentiation [39]. ALP staining was performed as an indicator of osteogenic differentiation on Day 7 and 14 (both liquid and hydrogel phases) and Day 14 (hydrogel phase). The BCIP/NBT staining solution was prepared by dissolving one tablet in 10 mL of distilled water, stored in the dark, and used within 2 hours of preparation. The wash buffer was made by adding 0.05% Tween 20 to Dulbecco's PBS (free of Ca^{2+}/Mg^{2+}). Cells were removed from the incubator, and the culture medium was aspirated. They were gently washed with PBS, then fixed in 10% neutral buffered formalin for 60 seconds in a fume hood. After fixation, cells were washed again with PBS. The monolayer was then covered with BCIP/NBT staining solution and incubated in the dark at room temperature for 5–10 minutes, with color development monitored every 2–3 minutes. Once staining was complete, cells were rinsed with wash buffer, and PBS was added to keep the samples hydrated. ALP activity was visualized under the microscope. ImageJ software was used to quantify the staining intensities.

2.7. Statistical analysis

Experiments were performed in triplicate using three independent biological replicates (n = 3). Data are expressed as mean ± standard deviation (SD). Statistical significance was assessed using two-way ANOVA, with treatment and time as factors, followed by Holm-adjusted multiple comparisons of each treatment with the corresponding control at each time point. * Indicates $p < 0.05$, and ** indicates $p < 0.01$. Analyses were conducted using IBM SPSS Statistics software.

3. Results

3.1. LA-GAGR and Mini-GAGR induces higher calcium deposition compared to other polysaccharides

Calcium deposition by cells cultured with the five polysaccharides, LA-GAGR, Mini-GAGR, HA, WG, and dextran, was evaluated using ARS staining. It is a sensitive method that chelates calcium ions deposited in the ECM, serving as an indicator of mineralization and osteogenic differentiation. The intensity and distribution of the resulting red-orange staining reflect the extent and localization of calcium deposition. Pre-osteoblasts were cultured with each polysaccharide and compared to untreated controls to assess their osteoinductive potential. ARS staining showed greater calcium deposition with LA-GAGR and Mini-GAGR at Days 7 and 14 (Figure 1A,B), indicating enhanced mineralization. Notably, the hydrogel form of both LA-GAGR and Mini-GAGR showed increased calcium deposition compared to their liquid phase counterparts, particularly at Day 14 (Figure 1A), suggesting the hydrogel environment may influence the observed mineralization response.

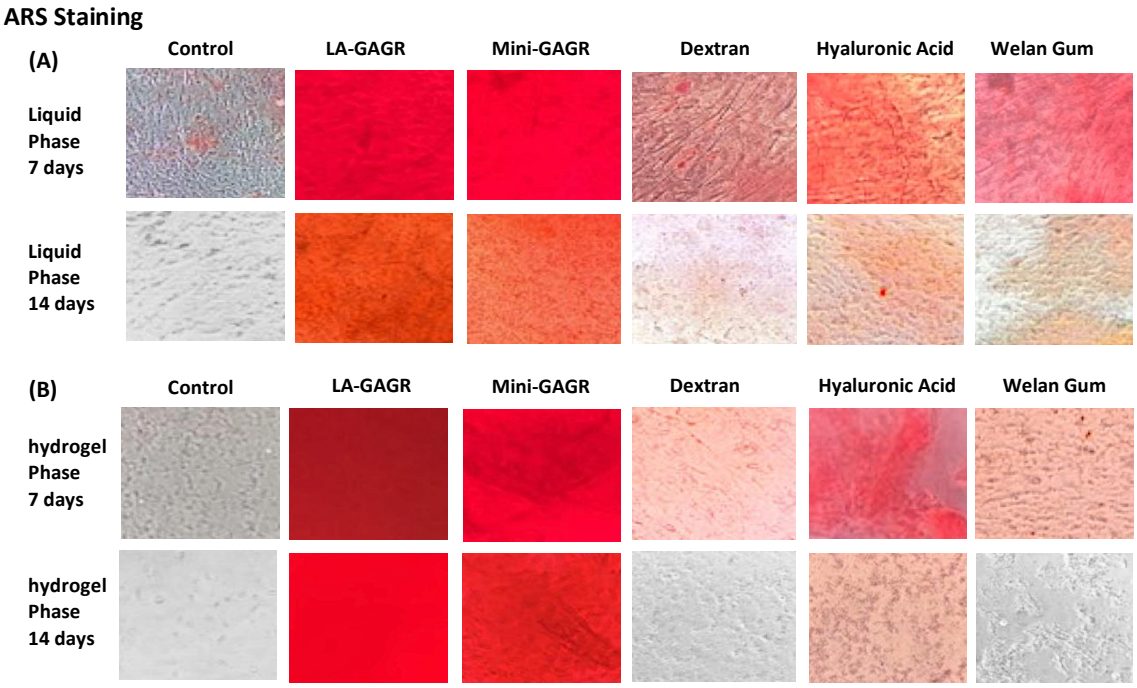


Figure 1. Images of ARS of calcium deposition in cells treated with different polysaccharides (LA-GAGR, Mini-GAGR, HA, WG, and dextran) compared with the control group after 7 and 14 days of culture. Results are presented for both liquid (Figure 1A) and hydrogel (Figure 1B) phases. LA-GAGR and Mini-GAGR exhibited the most intense staining at both time points, indicating greater calcium deposition and enhanced mineralization compared with the other treatments.

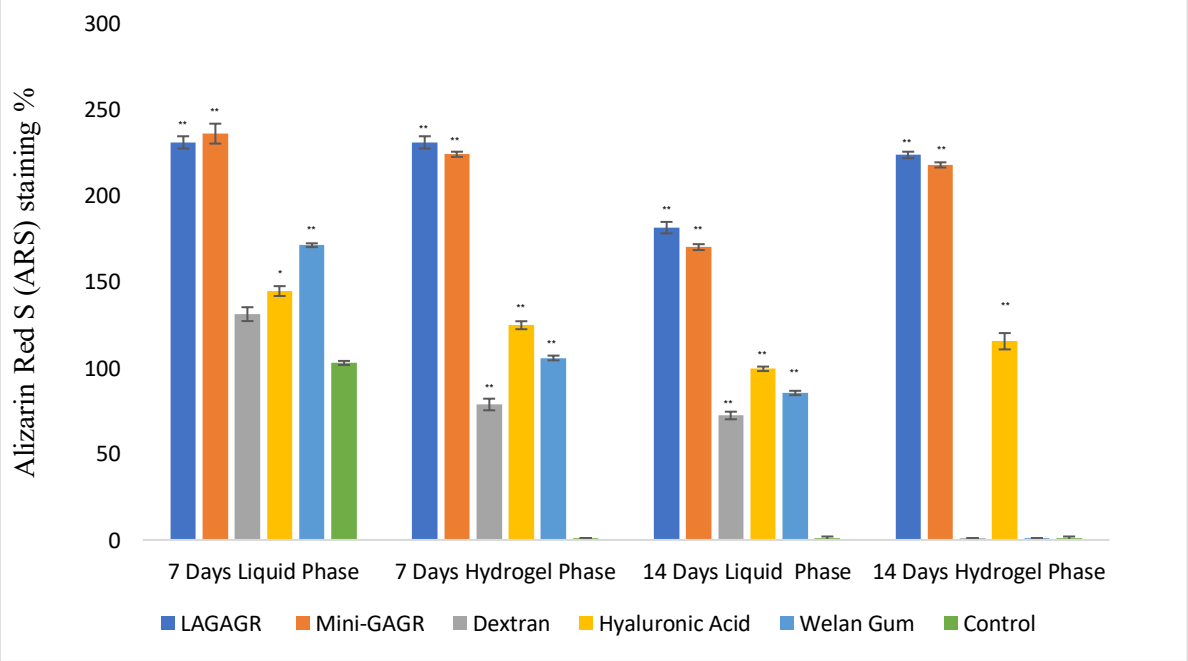


Figure 2. Quantitative analysis of ARS staining intensity for polysaccharide-treated samples at 7 and 14 days. ARS staining is presented as a percentage relative to the control group, providing a comparative assessment of calcium deposition and matrix mineralization among treatments. Data are presented as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA compared with the control (*$p < 0.05$, **$p < 0.01$).

These ARS results showed differences in staining intensity across the treatment groups and time points. Quantitative analysis of staining intensity, extracted and measured for each condition (Figure 2), confirmed that LA-GAGR and Mini-GAGR, particularly in hydrogel form, induced higher calcium accumulation at both time points compared to HA, WG, and dextran. These findings are consistent with the qualitative observations from Figure 1 and suggest a greater mineralization-associated response in the GAGR-treated groups under the tested conditions.

3.2. LA-GAGR exhibits osteogenic potential as indicated by ALP staining

To reconfirm the osteogenic potential of the polysaccharides used in this study, ALP staining has been used as a measure of Alkaline phosphatase activity. As shown in Figures 3 and 4, MC3TC3 cells treated with LA-GAGR, especially in the hydrogel phase, exhibited markedly higher ALP staining intensity compared to other treatments at both Day 7 and Day 14. No ALP staining was observed in Mini-GAGR and other polysaccharide-treated groups, as well as the control. Interestingly, the staining intensity results on Day 7 liquid phase show moderate ALP expression with addition of WG (Figure 4), which could be due to its structural similarity with GG [31]. The higher ALP staining intensity observed with LA-GAGR is consistent with its potential to support early osteoblastic differentiation and complements the ARS findings.

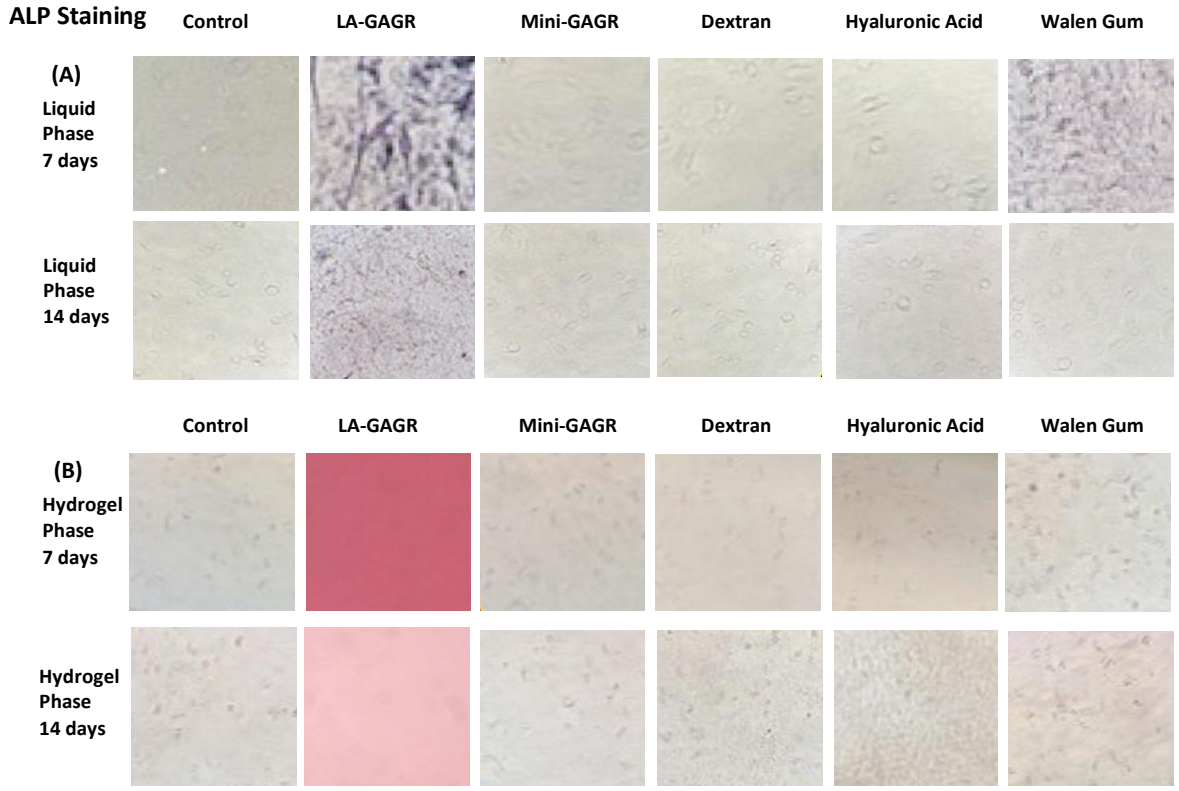


Figure 3. Image of ALP staining of cells treated with different polysaccharides (LA-GAGR, Mini-GAGR, HA, WG, and dextran) compared with the control group after 7 and 14 days of culture. Results are presented for both liquid (Figure 3A) and hydrogel (Figure 3B) phases. LA-GAGR showed the most intense staining at both time points, indicating higher ALP activity and enhanced early osteogenic differentiation relative to other treatments.

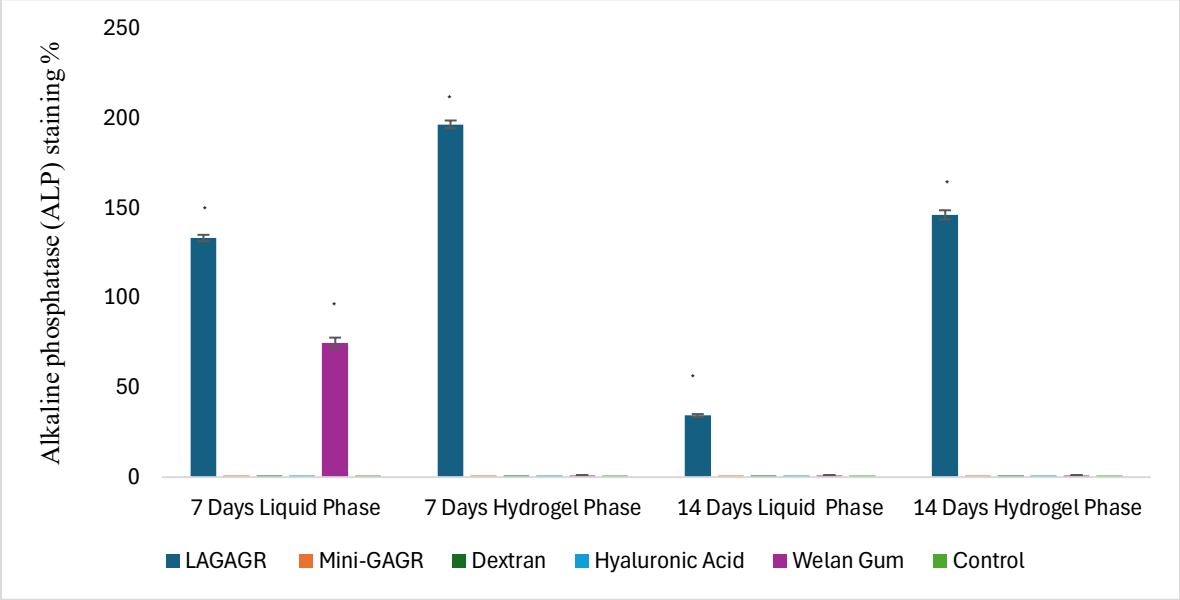


Figure 4. Quantitative analysis of ALP staining intensity for polysaccharide-treated samples at 7 and 14 days. ALP activity is presented as a percentage relative to the control group, providing a comparative assessment of osteogenic activity among treatments. Data are presented as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA compared with the control (*$p < 0.01$).

3.3. Hydrogel phase enhances osteogenic differentiation in both GG variants

Hydrogels provide a three-dimensional environment that supports cellular functions essential for bone regeneration. Their porous architecture facilitates cell adhesion and migration while enabling efficient exchange of nutrients, oxygen, and metabolic waste, conditions critical for osteogenic differentiation [40,41] In our study, the hydrogel formulations of LA-GAGR and Mini-GAGR showed greater calcium deposition and ALP activity than their corresponding liquid formulations at Days 7 and 14 (Figures 1–4). These results indicate that the GG derivatives can support osteogenic differentiation and ECM mineralization. While HA, WG, and dextran also exhibited some variation between phases, the most consistent osteogenic responses were observed in the LA-GAGR and Mini-GAGR groups across both time points. For HA, WG, and dextran, Day 7 results in the liquid phase showed slightly higher activity than the hydrogel form.

Overall, the results suggest that hydrogel environments provide more favorable conditions for osteogenic differentiation than liquid-phase cultures. In this context, a clear molecular-weight-dependent difference was observed between the two GG variants, particularly with respect to ALP staining and expression of stage-associated osteogenic markers. Although both LA-GAGR and Mini-GAGR enhanced calcium deposition and osteogenic gene expression, only LA-GAGR exhibited pronounced ALP activity, whereas Mini-GAGR showed no detectable ALP response. This distinction could be attributed to the molecular which are further discussed in Section 3.4.3.

3.4. Osteogenic gene expression patterns

Gene expression analysis showed an upregulation of osteogenesis-related markers from Day 3 to Day 14 across all polysaccharide treatments compared to the control (Figures 5, 6, 7, and 8). Osteogenic

differentiation markers such as RUNX2 and SP7 demonstrated upregulation from Day 3 to Day 7 followed by stabilization by Day 14. In contrast, the late-stage markers OCN, COL1, and OPN showed low expression at Day 3, but demonstrated a consistent and marked increase, peaking at Day 14.

Among the tested polysaccharides, LA-GAGR and Mini-GAGR induced the highest expression levels of early-stage genes, highlighting their potential to promote osteoblastic differentiation.

3.4.1. Early osteogenic commitment is characterized by high RUNX2 and SP7 expressions

The expression levels of RUNX2 and SP7 (Osterix), two key transcription factors that regulate early osteoblast differentiation, were assessed via qRT-PCR [42]. RUNX2, recognized as the master regulator of osteoblast lineage commitment, regulates the expression of key osteogenic genes, including COL 1, OCN, and OPN, in pre-osteoblasts and enhances matrix maturation and mineralization by upregulating ALP activity [43]. SP7, acting downstream of RUNX2, is essential for driving pre-osteoblasts toward maturation into fully functional osteoblasts, thereby supporting bone matrix formation and mineral deposition [44]. Consistent with their known biological roles, RUNX2 and SP7 expressions were significantly upregulated between Day 3 and Day 7, suggesting an early osteogenic response (Figure 5 A,B). Both RUNX2 and SP7 share regulatory roles over osteoblast-specific genes including those involved in matrix synthesis and mineralization. Most of these gene promoters contain binding motifs for both transcription factors, suggesting their complementary and synergistic function during the osteogenic process [45].

At Day 3, RUNX2 expression (Figure 5A), was relatively low across all treatments, with slightly higher levels observed in Mini-GAGR (~5.3-fold increase in relative expression) and LA-GAGR (~4.8-fold increase). By Day 7, expression peaked sharply in Mini-GAGR and LA-GAGR (~9.5 and ~7.6-fold increase, respectively). At Day 14, it remained elevated in Mini-GAGR (~12.7-fold increase) and LA-GAGR (~8.5-fold increase). HA and WG-treated cells also showed a significant increase in expression by Day 14 (~6.7- and 5.4-fold increase respectively), while cells treated with dextran exhibited minimal induction throughout the three time-points.

SP7 expression (Figure 5B) showed a similar trend, rising from low levels at Day 3 to slightly higher at Day 7 in Mini-GAGR (~9-fold increase) and LA-GAGR (~4.5-fold increase). By Day 14, expression levels continue to rise with Mini-GAGR showing a ~15.3-fold increase and LA-GAGR showing a ~11.7-fold increase. In contrast, WG and HA showed only modest increases. The concurrent increase in RUNX2 and SP7 expression support the notion that Mini-GAGR and LA-GAGR may potentially induce early osteogenic commitment, likely through upregulation of these transcription factors.

Additionally, SOX9 is another early-stage transcription factor that plays a complex role. It facilitates the differentiation of MSCs into the osteochondroprogenitor cells and further differentiation into chondrocytes. It also suppresses osteogenic differentiation by directly interfering with RUNX2 function. Downregulation of SOX9 is essential for osteoblast lineage progression. Our data show that SOX9 expression (Figure 5C) declined in polysaccharide-treated cells, especially with LA-GAGR (from ~5.2-fold increase at Day 3 to ~2.6 fold at Day 7 and ~2.4-fold at Day 14) reinforcing the shift toward the osteogenic pathway and confirming osteocyte lineage specification. With Mini-GAGR also SOX9 expression fell from ~3.7-fold to ~2-fold by Day 7 but rose modestly to 3-fold at Day 14. HA showed the highest early induction (~7.3-fold increase) followed by a progressive decrease to 4.7-fold by Day 14, whereas WG showed moderately elevated levels (from 2.6-fold increase at Day 3 to ~3.3-fold at Day 7 and 14). Dextran and the control remained near baseline (< 1.9-fold increase throughout). Collectively,

the pronounced down-regulation in LA-GAGR, and to a lesser extent Mini-GAGR and HA, is consistent with a shift toward an osteogenic phenotype.

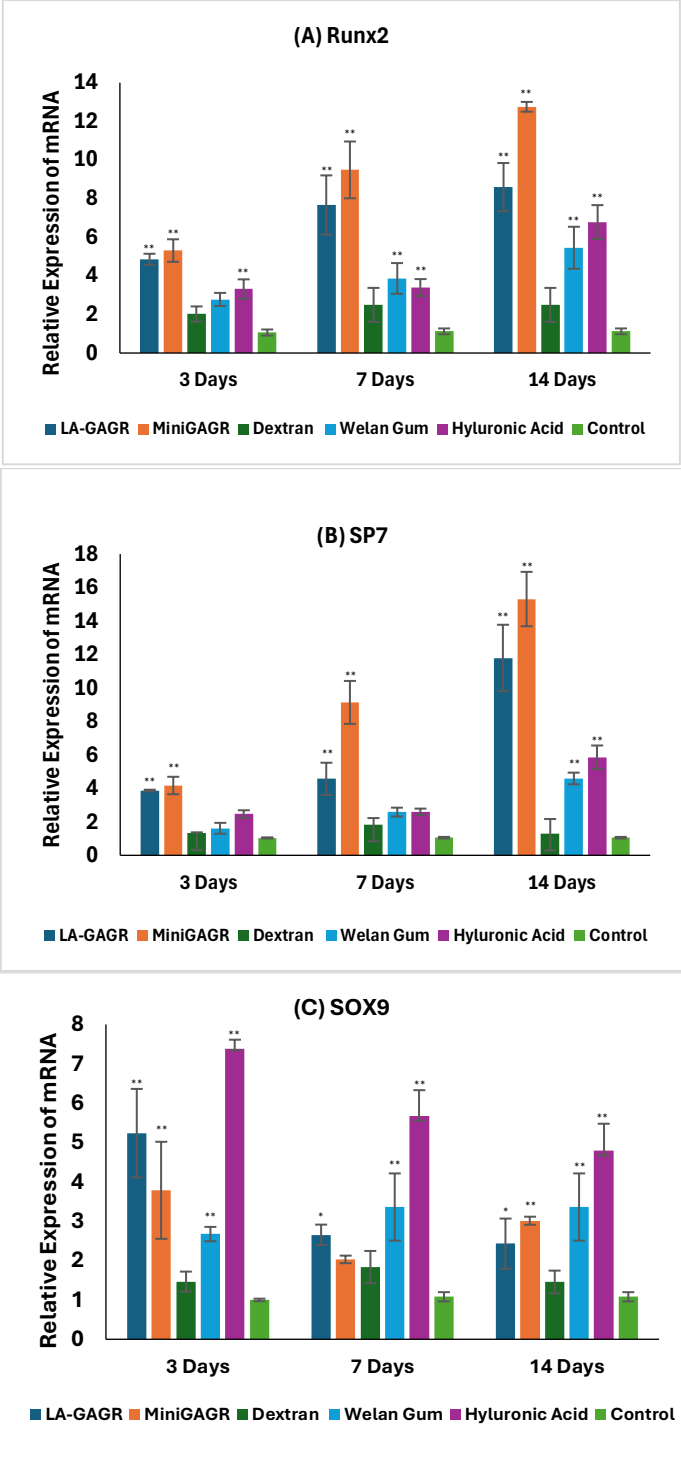
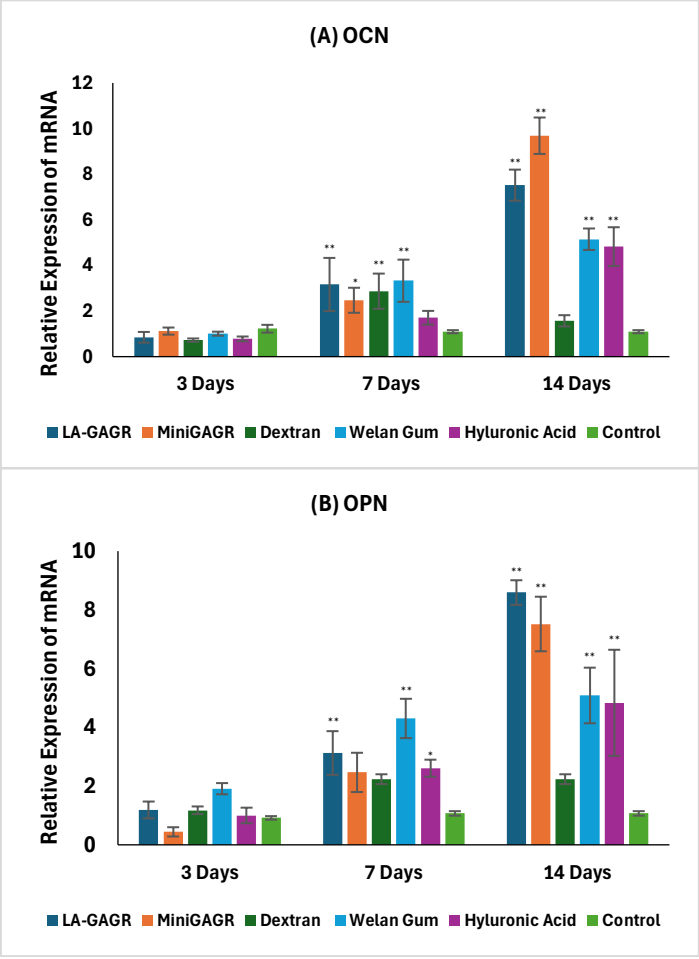


Figure 5. Gene expression analysis of early-stage osteogenic genes. **(A)** Runx2; **(B)** SP7; and **(C)** SOX9 at days 3, 7, and 14 in MC3T3-E1 cells cultured with different polysaccharides. The results show time-dependent changes in gene expression, indicating the potential of these biomaterials to regulate early osteogenic differentiation. Data are presented as mean ± SD (n = 3). Statistical significance was assessed using two-way ANOVA with treatment and time as factors, followed by Holm-adjusted multiple comparisons of each treatment with the corresponding control at each time point. $p < 0.05$ and $p < 0.01$.

3.4.2. Matrix-associated genes are upregulated in GAGR-treated groups

OCN expression (Figure 6A) showed a progressive increase over time in all polysaccharide groups, with particularly high induction in Mini-GAGR and LA-GAGR-treated cells. On Day 3, OCN levels remained low across all samples (~0.7-1.2-fold increase), indicating early-stage differentiation. However, by Day 7, expression had increased to ~2-3-fold across all treatment groups and continued to increase by Day 14 reaching ~7.5-fold with LA-GAGR and ~9.5-fold with Mini-GAGR. WG and HA also showed significant upregulation by Day 14 (~5-fold increase), but to a lesser extent compared to GAGR. Dextran and control groups maintained relatively low expression levels.

OPN expression (Figure 6B) increased in all groups following a similar trend, with the highest levels observed in GAGR-treated cells. On Day 3, expression was modest across most groups (< 2-fold increase). Mini-GAGR showed the lowest expression at this time point (~0.4-fold increase), whereas WG showed a highest (~1.9-fold). By Day 7, gene expression increased significantly in all groups, with WG showing the highest levels (~4.3-fold), followed by LA-GAGR (~3.13-fold), HA (~2.61-fold), and Mini-GAGR (~2.47-fold). By Day 14, OPN expression reached ~8.6-fold increase in LA-GAGR and ~7.5-fold increase in Mini-GAGR. HA and WG also showed substantial increases (~4.8-fold and ~5.1-fold, respectively), though to a lesser extent. In contrast, dextran and control group remained relatively unchanged across time points.



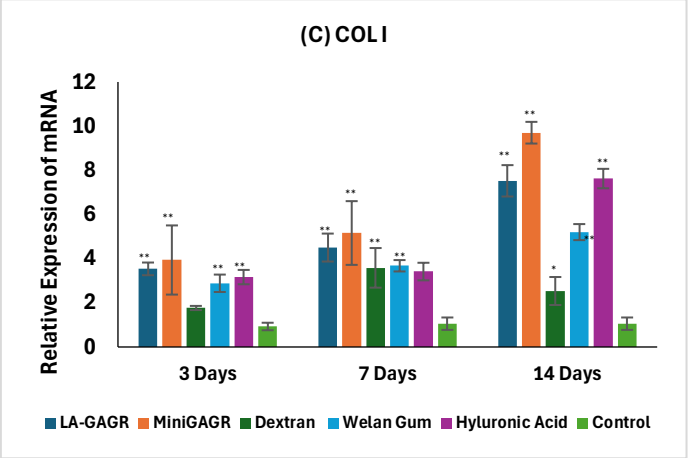


Figure 6. Gene expression analysis of extracellular matrix–associated genes. **(A)** OCN; **(B)** OPN; and **(C)** COL1 at days 3, 7, and 14 in MC3T3-E1 cells cultured with different polysaccharides. The results show time-dependent changes in the expression of these markers, highlighting the potential of these biomaterials to influence matrix maturation during osteogenesis. Data are presented as mean ± SD (n = 3). Statistical significance was assessed using two-way ANOVA with treatment and time as factors, followed by Holm-adjusted multiple comparisons of each treatment with the corresponding control at each time point. $p < 0.05$ and $p < 0.01$.

COL1 expression (Figure 6C), a marker for ECM, was also upregulated in Mini-GAGR and LA-AGR treatment groups. On Day 3, Mini-GAGR and LA-GAGR showed the highest expression (~3.9-fold and ~3.5-fold increase, respectively), followed by HA (~3.1-fold) and WG (~2.8-fold). In contrast, dextran and control groups exhibited comparatively lower levels (~1.7-fold and ~0.9-fold, respectively). By Day 7, expression further increased in GAGR-treated cells, with Mini-GAGR reaching ~5.1-fold and LA-GAGR ~4.5-fold, while HA and WG maintained moderate levels (~3.4–3.7-fold). At Day 14, COL1 expression peaked at ~9.7-fold in Mini-GAGR and ~7.5-fold in LA-GAGR. HA and WG also exhibited significant increases (~7.6-fold and ~5.2-fold, respectively). In comparison, expressions in dextran group declined to ~2.5-fold, and the control group remained unchanged (~1-fold). The elevated COL1 expression in GAGR groups is consistent with enhanced expression of other ECM-associated genes during osteogenic differentiation.

3.4.3. LA-GAGR and Mini GAGR induces early VEGF expression during osteogenic differentiation

VEGF expression (Figure 7) was found to peak at Day 3 across all treatment groups, suggesting an early increase in VEGF-associated signaling. Notably, Mini-GAGR (~9.1-fold increase) and dextran (~7.5-fold increase) showed the highest expression, followed by LA-GAGR (~6.2-fold increase), indicating a strong pro-angiogenic signal in the early phase. HA (~3.8-fold increase) and WG (~2.9-fold increase) also showed moderate expression compared to the control (~0.9-fold increase). By Day 7, expression levels declined in all groups, although Mini-GAGR and dextran maintained relatively higher levels (~3.9-fold and ~5.3-fold increase, respectively). Expression in LA-GAGR and the other treatment groups decreased further, and this downward trend through Day 14. The early surge followed by a gradual decline by Day 7 and Day 14, is consistent with the transient role of VEGF in early neovascularization during bone repair [46]. Hence, the VEGF gene expression pattern suggests that Mini-GAGR and LA-GAGR may promote pro-angiogenic signaling during the osteogenesis.

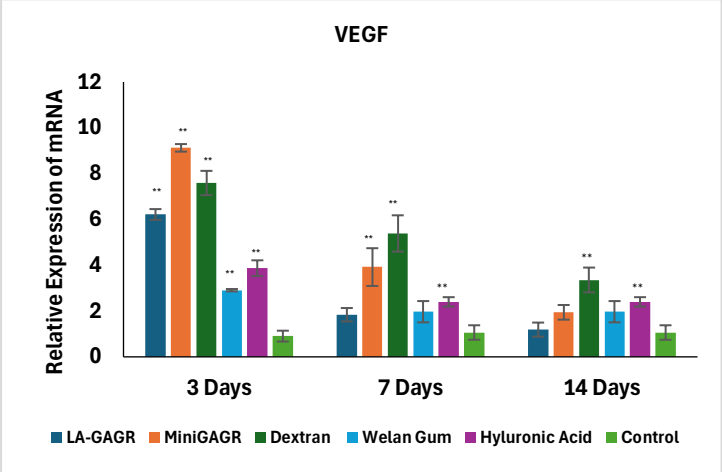


Figure 7. Gene expression analysis of VEGF at days 3, 7, and 14 in MC3T3-E1 cells cultured with different polysaccharides. The results show a time-dependent increase in VEGF expression, indicating the potential of these biomaterials to modulate early angiogenic signaling during osteogenesis. Data are presented as mean ± SD (n = 3). Statistical significance was assessed using two-way ANOVA with treatment and time as factors, followed by Holm-adjusted multiple comparisons of each treatment with the corresponding control at each time point. $p < 0.05$ and $p < 0.01$.

A summary of the relative fold-change expression of key osteogenic genes is shown in Table 2. It is evident that Mini-GAGR exhibits higher expression levels across most time points and gene targets, particularly for early-stage and ECM-associated markers, including RUNX2, SP7, COL1, OCN, and VEGF-A. In contrast, LA-GAGR shows relatively greater upregulation of OPN, a late-stage matrix proteins, suggesting the two variants may elicit distinct osteogenic responses. Compared with the liquid phase, LA-GAGR and Mini-GAGR hydrogels demonstrated higher osteogenic responses in this study.

Table 2. Comparison of relative fold-change expression of osteogenic genes treated with LA-GAGR and Mini-GAGR. Bold values indicate the higher fold-change expression between LA-GAGR and Mini-GAGR at each time point.

Gene	Day	LA-GAGR (Fold Change)	Mini-GAGR (Fold Change)	Trend/Interpretation
RUNX2	3	~4.8×	**~5.3×**	Mini > LA (early activation)
	7	~7.6×	**~9.5×**	Mini > LA
	14	~8.5×	**~12.7×**	Mini > LA (peak expression)
SP7 (Osterix)	3	~2.1×	**~3.6×**	Mini > LA (early osteogenic signal)
	7	~4.5×	**~9.0×**	Mini > LA
	14	~11.7×	**~15.3×**	Mini > LA (maturation phase)
COL1	3	~3.5×	**~3.9×**	Mini > LA (early ECM formation)
	7	~4.5×	**~5.1×**	Mini > LA
	14	~7.5×	**~9.7×**	Mini > LA (peak ECM deposition)
OPN	3	~1.2×	~0.4×	Both low early
	7	**~3.1×**	~2.5×	LA > Mini (mid-stage increase)
	14	**~8.6×**	~7.5×	LA > Mini (late matrix protein)
OCN	3	~1.0×	~1.2×	Both low (early stage)
	7	~2.5×	~3.0×	Mini > LA (onset of mineralization)
	14	~7.5×	**~9.5×**	Mini > LA (active mineral deposition)
SOX9	3	**~5.2×**	~3.7×	LA > Mini (initial elevation; chondrogenic marker)
	7	**~2.6×**	~2.0×	Both decreased (osteogenic shift)
	14	**~2.4×**	~3.0×	LA lower; stronger commitment to osteogenesis
VEGF	3	~6.2×	**~9.1×**	Mini > LA (early pro-angiogenic signal)
	7	~2.7×	**~3.9×**	Mini > LA (sustained angiogenic support)
	14	~1.0×	~1.2×	Both near baseline (transient expression phase)

4. Discussion

Our results show that both GG derivatives promoted an osteogenic response in pre-osteoblast MC3T3-E1 cells, as indicated by increased ARS staining, ALP staining intensity with LA-GAGR, and changes in the *in vitro* transcriptional expression of osteoinductive genes (RUNX2, SP7) and matrix-related genes (OCN, OPN, COL1). Compared to HA, WG, and dextran, both GG variants showed higher ARS staining intensity, consistent with greater matrix mineralization. Interestingly, LA-GAGR showed higher ALP staining intensity. Mini-GAGR demonstrated strong ARS staining and showed the highest expression of RUNX2, COL1, OPN, and SP7 across most time-points, along with elevated VEGF expression on day 3, when compared to LA-GAGR and the other polysaccharides. These findings suggest that Mini-GAGR may support osteogenic differentiation and matrix mineralization under the tested conditions.

The hydrogel formulation of each polysaccharide demonstrated enhanced osteogenic activity in comparison to liquid formulation, possibly suggesting the requirement of a three-dimensional scaffold environment that more closely mimics native bone-forming conditions [11]. Gene expression data further support the osteogenic capacity of LA-GAGR and Mini-GAGR. Expression of the early osteogenic markers RUNX2 and SP7 increased by Day 7, followed by higher expression of the matrix-associated genes OCN, OPN, and COL1 at Day 14. Both GG variants also showed an early increase in VEGF expression, which peaked at Day 3. Research has shown that the expression of genes such as VEGF and RUNX2 plays a critical role in driving vascular invasion during bone regeneration, with studies demonstrating enhanced angiogenesis and bone formation when these factors are co-administered, for example in mandibular defect models [47]. Interestingly, recent studies indicate that a specific VEGF dose range is essential for coupling vascular invasion with bone formation [48]. The ability to support both osteogenesis and angiogenesis highlights the multifunctional role of GG scaffolds in bone regeneration. In the present study, the early increase in VEGF expression suggests that GG derivatives may provide pro-angiogenic signaling during the early stages of osteogenic differentiation.

The inclusion of both LA-GAGR and Mini-GAGR in this study was intended to elucidate how molecular weight influences the osteogenic and angiogenic responses of GG-based biomaterials. Despite their shared chemical backbone, Mini-GAGR, with its lower molecular weight (760 Da), demonstrated higher expression of the early osteogenic markers (RUNX2 and SP7) and the matrix-associated genes (COL1 and OCN), as well as the calcium deposition observed in ARS staining. In contrast, LA-GAGR, with its higher molecular weight, showed higher ALP activity. These differences may be associated with the distinct molecular characteristics of the two GG derivatives; however, the present study did not directly investigate the mechanisms underlying the molecular-weight-dependent responses. Such molecular-weight-dependent behavior has been reported previously for other polysaccharides, particularly HA. Prior studies demonstrated that low-molecular-weight HA significantly enhanced cell proliferation and osteogenic gene expression, including OCN mRNA, yet did not induce alkaline phosphatase activity. In contrast, high-molecular-weight HA markedly increased ALP activity in a dose-dependent manner [49]. This observation can be explained by the stage-dependent nature of osteogenic differentiation. ALP is one of the early markers of osteogenic differentiation, whereas calcium deposition represents a later stage of mineralization. Importantly, previous studies have demonstrated that ALP activity can be dissociated from the expression of late-stage osteogenic markers such as OCN and OPN, and that cells with reduced ALP activity can still differentiate into a mineral-secreting

phenotype. Cell lines exhibiting severe repression of ALP activity differentiate to a mineral-secreting phenotype much like normal MC3T3-E1 cells [49]. In line with this decoupling, Mini-GAGR may accelerate progression toward a mature, mineral-secreting phenotype without requiring sustained early ALP activity. Therefore, the absence of ALP activity does not necessarily indicate reduced osteogenic potential. Consistent with this pattern, Mini-GAGR, despite lacking detectable ALP activity, strongly enhanced osteogenic gene expression including high expression of the late-stage markers osteocalcin (OCN) and osteopontin (OPN) as well as calcium deposition evidenced by robust ARS staining. Furthermore, it enhanced VEGF expression. Collectively, these findings suggest that Mini-GAGR may influence osteogenic commitment and differentiation processes. Further protein-level studies will be needed to clarify the mechanisms involved.

Additionally, as shown in Figures 2 and 4, the ARS and ALP results at 7 and 14 days reveal time-dependent differences between liquid and hydrogel conditions. At day 7, LA-GAGR and Mini-GAGR exhibited slightly higher ARS intensity in the liquid condition compared to the hydrogel; however, this mineralization response declined more rapidly in the liquid phase by day 14. In contrast, the hydrogel condition supported more sustained ARS and ALP activity over the culture period. The enhanced and sustained ARS and ALP responses observed under hydrogel conditions reflect influence of three-dimensional matrix on cellular responses [50]. However, the specific factors contributing to these differences were not investigated in the present study.

The superior performance of GG variants over other polysaccharides may be attributed to not only to their structural characteristics but also to differences in surface charge density, and chain conformation which collectively influence bone cell interactions associated with osteogenic differentiation. GG contains L-rhamnose residues, which are absent in both (HA Figure 8D) and dextran (Figure 8E) [51,52]. Structurally, LA-GAGR and Mini-GAGR are composed of repeating tetrasaccharide units consisting of glucose, glucuronic acid, and rhamnose, resulting in a negatively charged polysaccharide (Figure 8A and B, respectively). In contrast, HA is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine (Figure 8D). HA has been widely studied for its osteogenic effects [51], with prior reports demonstrating that HA primarily modulates osteogenesis at the transcriptional level by influencing the expression of osteogenesis-related genes [49].

This behavior is consistent with the present findings, in which HA exhibited modest gene expression responses compared with the more pronounced and sustained osteogenic effects observed for LA-GAGR and Mini-GAGR. Dextran, in contrast, is a neutral polysaccharide composed of repeating α-D-glucopyranose units (Figure 8E). Previous studies have reported that dextran exerts minimal effect on osteogenic differentiation, including ALP activity, matrix mineralization, and the expression of osteogenic markers such as COL1, OPN, and OCN [53]. This is consistent with the present results, where dextran showed relatively low osteogenic activity in both ARS and ALP assays. However, it can be noted that dextran induced a pronounced early upregulation of VEGF expression in the current study. This aligns with previous studies reporting that dextran can promote endothelial differentiation and VEGF-associated angiogenic signaling [54]. This also suggests that dextran may preferentially support pro-angiogenic signaling response. WG shares a similar backbone structure with GG, composed of repeating tetrasaccharide units, but contains additional L-rhamnosyl or L-mannosyl side chains substituted at the C3 position of the first glucose residue (Figure 8C) [55]. The differences in polysaccharide side-chain substitutions, surface charge distribution, and molecular conformation may influence protein binding or

receptor-mediated interactions in biological environments [56,57]. Although WG demonstrated some osteogenic response, its branched architecture distinguishes it from GG variants and may contribute to the differences observed in biological effects.

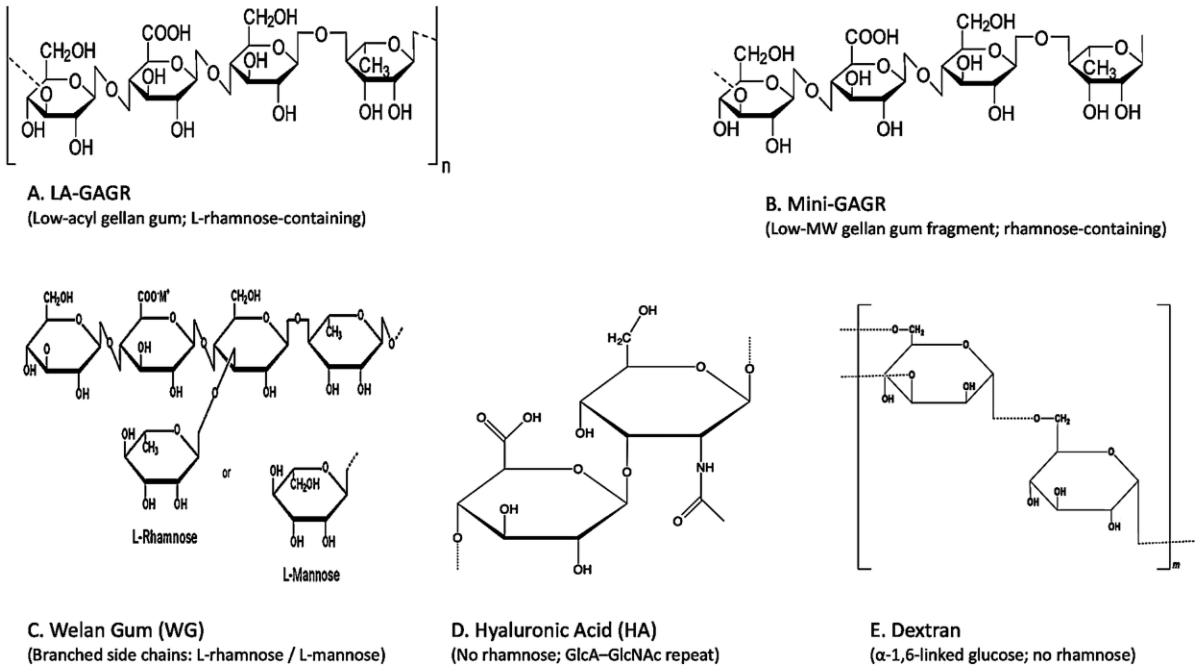


Figure 8. Chemical structures of polysaccharides evaluated in this study. **(A)** Low-acyl gellan gum (LA-GAGR); **(B)** Mini-GAGR; **(C)** Welan gum showing branched side chains; **(D)** Hyaluronic acid; **(E)** Dextran. The presence of L-rhamnose residues in GG variants and WG, and the absence of rhamnose in HA and dextran.

In addition to differences in structure, the presence of L-rhamnose residues in GG, which have been associated with bioactive effects, may also influence osteoblast differentiation. Rhamnose-rich oligo- and polysaccharides (RROPs) have been reported to provide cytoprotective and anti-inflammatory effects, which create a favorable environment for osteogenesis. Specifically, RROPs stimulate cell proliferation, enhance collagen production, protect hyaluronan from oxidative damage, and also reduce elastase activity [58]. These properties could help explain the enhanced calcium deposition, RUNX2, COL1a and OCN expression observed in our Mini-GAGR and LA-GAGR groups. In addition, their ability to mitigate advanced glycation end-products (AGEs)-induced cytotoxicity suggests that rhamnose-containing polymers may be particularly beneficial in compromised environments, such as diabetic bone healing. Taken together, these findings support the idea that the presence of L-rhamnose residues may play a role in the biochemical signaling of GG-based materials. In a study by Faury *et al.*, cell proliferation and ECM biosynthesis were found to be mediated via α-L-rhamnose-specific lectin receptors on human skin fibroblasts and keratinocytes. Upon ligand binding, RROPs activated intracellular calcium signaling in a dose-dependent manner and altered the expression of genes involved in growth, adhesion, and extracellular matrix remodeling [59]. These observations raise the possibility that the L-rhamnose in GG-derived polysaccharides may interact with similar receptors in osteoblasts or their progenitors. Such interactions could potentially be involved in calcium-dependent signaling cascades that could promote osteogenic differentiation. Although the exact receptors and pathways in

bone-forming cells remain to be identified, present evidence suggests that GG scaffolds could exert biological effects. Despite these promising findings, knowledge gaps remain. The distinct roles of molecular weight, structural conformation, and charge distribution in modulating GG-cell interactions require further investigation. Furthermore, the downstream signaling mechanisms activated by GG cell engagement warrant detailed mechanistic studies to fully elucidate their role in bone regeneration.

Our present study showing increased osteogenic responses with LA-GAGR and Mini-GAGR hydrogels are particularly noteworthy given their chemically simple, polysaccharide-only composition and their ability to elicit substantial upregulation of osteogenic markers at the transcriptional level. This contrasts with some recently published multifunctional scaffolds that achieve enhanced bone regeneration through more complex design strategies. For example, phosphorus-dendron-functionalized electrospun nanofibrous sponges use dynamic Cu^{2+} capture and pH-responsive release to promote neovascularization and osteogenic differentiation [60]. More recently, a calcitonin gene-related peptide (CGRP)-empowered composite delivery system was developed incorporating CGRP-loaded microspheres, stem cell sheets, and modified nanofiber sponges to enhance neurovascular coupling and promote coordinated neural, vascular, and bone regeneration [61]. In comparison, LA-GAGR and Mini-GAGR hydrogels showed increased osteogenic responses without added metal ions or exogenous cell populations, suggesting that the polymer matrix itself contributes to the observed bioactivity. Although the molecular basis of this activity remains to be established, these findings position GG derivatives as potential base matrices for further functionalization with additional inorganic or biological cues, such as Sr^{2+}, Cu^{2+}, ceramics or stem cells.

Several limitations should be considered when interpreting the present findings. First, the polysaccharides were evaluated at formulation-specific rather than concentration-matched concentrations. For the hydrogel formulations, LA-GAGR, Mini-GAGR, HA, dextran, and WG were used at 1.0%, 3.0%, 1.0%, 7.0%, and 1.0% (w/v), respectively. Therefore, concentration-dependent effects cannot be excluded. Optimization of the concentrations of each polysaccharide will therefore be important in future comparative studies. Second, ALP and ARS were assessed from staining intensity and were not normalized to cell number or total protein; therefore, these measurements provide semi-quantitative rather than direct measures of ALP activity or calcium content. Finally, protein-level validation and detailed hydrogel characterization were beyond the scope of this initial comparative study. Future studies incorporating quantitative biochemical assays, protein-level analysis, and comprehensive hydrogel characterization will further validate the observed osteogenic responses.

5. Conclusion

This study demonstrates that both LA-GAGR and Mini-GAGR exhibit higher osteogenic potential, compared directly with other natural polysaccharides, as reflected by changes in osteogenic gene expression, ALP staining, and ECM mineralization. Both variants upregulated key osteogenic genes and showed an early increase in VEGF expression which suggests potential pro-angiogenic signaling. These results highlight their potential as a bioactive material for bone tissue engineering applications. The comparative gene expression results are summarized in Table 2. Mini-GAGR exhibited consistently higher expression than LA-GAGR of early osteogenic genes and matrix-forming genes, including RUNX2 (≈ 49%), SP7 (≈ 31%), COL1 (≈ 29%), OCN (≈ 27%), and VEGF-A (≈ 47%), whereas LA-GAGR exhibited greater upregulation of OPN (≈ 15%) along with enhanced ALP staining intensity. Both variants

demonstrated enhanced osteogenic and VEGF gene expression compared with the other polysaccharides, under the tested conditions. Together with their high biocompatibility and tunable gelation properties, these findings position GG variants as promising candidates for functional biomaterials in bone tissue engineering.

Data availability statement

No supplementary or additional data were generated in this study.

Declaration of generative AI and AI-assisted technologies

During the preparation of this manuscript, the authors used ChatGPT for language editing and improving the clarity and readability of the text. The authors reviewed and edited the output and take full responsibility for the content of the manuscript.

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Authors' contribution

Conceptualization, M.A. and D.S.K.; methodology, M.A. and F.A.; software, M.A.; formal analysis, M.A.; investigation, M.A., D.S.K. and F.A.; resources, M.A. and D.S.K; data curation, M.A.; writing—original draft preparation, M.A., D.S.K. and F.A.; writing—review and editing, M.A., D.S.K. and F.A.; visualization, M.A., D.S.K. and F.A.; supervision, D.S.K.; funding acquisition, D.S.K; All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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