

Implementing BMP-7 Chemically Modified RNA for Bone Regeneration with 3D Printable Hyaluronic Acid-Collagen Granular Gels

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Chemically modified RNA (cmRNA) is emerging as a more effective alternative to protein delivery and DNA-based gene therapy. To implement this technology for bone regeneration, a suitable biomaterial functioning as scaffold and delivery system is necessary. This study introduces a 3D printable granular hydrogel consisting of hyaluronic acid and collagen (THA-Col) for the delivery of bone morphogenetic protein (BMP)-7 cmRNA as activated matrix to promote bone healing. Granular hydrogels are produced by mechanically fragmenting bulk THA-Col gels. Resulting microgels are 3D printable and are further investigated in comparison to bulk THA-Col gels for BMP-7 cmRNA transfection efficiency, cytotoxicity, and osteogenic differentiation of human mesenchymal stromal cells (hMSCs). Microgels showed higher cell viability than bulk gels, while both bulk and microgels could support transfection with BMP-7. During in vitro osteogenic differentiation, hMSCs on microgels showed higher production of alkaline phosphatase (ALP) compared to bulk gels. The combination of microgels loaded with BMP-7 cmRNA introduced in this work holds significant potential toward the development of patient-specific bone graft substitutes to replace autologous bone grafting and protein delivery.

in the case of large defects. Autologous bone grafting is still the current standard clinical treatment, making bone transplantation extremely prevalent.^[1] However, this procedure has major drawbacks, including donor site morbidity, limited quantity of bone that can be harvested for transplantation, and risk of infection or iatrogenic damage due to the invasive nature of the procedure.^[2] This highlights the need to develop alternatives to replace autografting as a treatment for bone defects.

Although biomaterials can support the osteogenic differentiation of human mesenchymal stromal cells (hMSCs),^[3] the incorporation of stimuli, such as growth factors, may significantly augment bone regeneration. Bone morphogenetic proteins (BMPs), like BMP-2 and BMP-7, are approved by European and North American authorities for multiple orthopedic applications.^[4–6] These proteins have a rather short half-life, and commercial products compensate for this by administering supraphysiological concentrations, which in turn has been associated with serious side effects.^[7,8]

The delivery of gene therapeutics encoding the growth factor of interest has gained popularity. For example, DNA-based gene

1. Introduction

With demographic shifts and increasingly active lifestyles, the incidence of bone fractures is steadily rising. Although bone possess an intrinsic healing capacity, this ability is often insufficient

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therapy can be implemented using vectors for delivery, allowing the own cell machinery to produce the desired protein. This has the advantage that proteins can be expressed for longer periods of time using lower administered doses. Messenger RNA (mRNA) offers numerous benefits compared to DNA-based therapies.^[9] These advantages include a more cost-effective and simpler production process for mRNA, enhanced efficiency in cell internalization and transfection, the absence of a requirement to enter the nucleus, and a reduced risk of mutations or genotoxic effects. The clinical success of SARS-CoV-2 vaccines is rapidly contributing to make this technology widely accessible.

Chemically modified RNAs (cmRNAs) are attracting rising interest due to increased stability and decreased immunogenicity compared to unmodified mRNA.^[10–13] BMP-2 cmRNA has been used for bone tissue engineering,^[12,14–18] including testing in a rat femoral defect, resulting in new bone formation after 2 weeks.^[16] BMP-2 cmRNA was also loaded in fibrin gel with calcium phosphate particles (CaP), to transfect hMSCs and induce osteogenesis.^[12] Our previous work delivering BMP-7 cmRNA to 2D cell monolayers indicates an osteogenic potential of this molecule.^[19] However, to the best of our knowledge, no research has been reported on creating a specific transcript-activated matrix for cmRNA delivery in bone regeneration.

Hydrogels have been widely used for bone tissue engineering and are excellent candidates for therapeutic delivery.^[20–22] Hydrogels mimic the natural extracellular matrix (ECM) environment and may promote biological activity. Many biomaterials have been used as hydrogels for bone tissue engineering, including natural and synthetic polymers.^[23] Natural biopolymers such as hyaluronic acid (HA) and collagen (Col) are popular due to their biocompatibility and abundance in natural ECM.^[24,25] HA is a carbohydrate polymer that can be modified with tyramine groups (THA) so that it can be independently crosslinked using enzymes and light, to produce a viscoelastic and shear-thinning hydrogel for 3D printing.^[26–28] HA has inherently limited cell attachment due to a lack of cell binding moieties, as well as being characterized with high swelling ratios.^[3] On the contrary, Col is rich in amino acid sequences like RGD that promote cell attachment and it is known for its cell-mediated shrinking.^[25,29] Therefore, the combination of THA and Col (THA-Col) can optimize cell shrinking and attachment, as demonstrated in bulk gels for supporting hMSCs in vitro chondrogenesis and osteogenesis.^[3,29]

Bulk hydrogels lack porosity, resulting in limited cell infiltration and migration. The use of microgels has emerged as a platform to generate microporous scaffolds^[30–33] and granular hydrogels have been demonstrated to support increased cell migration and proliferation compared to their bulk counterpart.^[33–35] Additionally, microgels have been reported to be excellent candidates for 3D printing due to their ability to flow under shear force.^[36,37] Microgels can be fabricated via batch emulsions, microfluidic emulsions, or mechanical fragmentation. Batch emulsion is straightforward and easily implementable, however it produces high polydispersity. Microfluidic emulsions offer high control over particle shape and size, however this comes with additional complexity, low yield, and challenging scalability.^[38,39] In mechanical fragmentation, microgels are produced by mechanically breaking a bulk hydrogel, for example by forcing it through a mesh. This method is simple and scalable, it does

not require the use of toxic materials, and microgel particle size and porosity can be controlled by varying the mesh size.^[40,41] In addition, granular hydrogels produced by fragmentation have been shown to support long-term cell culture, which has been attributed to higher mechanical stability compared to other microgel fabrication techniques.^[38] The use of mechanically fragmented microgels has gained popularity in recent years and many studies reported significant potential for different biological applications, such as zwitterionic granular hydrogels for cartilage repair^[41] and HA-based microgels for intervertebral disc repair.^[42] To our knowledge, granular hydrogels for therapeutic delivery of cmRNA to enhance bone repair have not yet been introduced.

Therefore, the goal of this study was to develop a 3D printable THA-Col granular hydrogel for the delivery of BMP-7 cmRNA to promote bone regeneration (**Figure 1**). To this aim, microgels were fabricated using mechanical fragmentation of bulk gels and compared to the exact same composition of unfragmented bulk gels. Bulk and microgels were characterized in terms of rheology, swelling, degradability, bulk mechanical properties, and microgels were also evaluated for printability. Cytotoxicity of THA-Col bulk and microgels was assessed in vitro via indirect toxicity using L929 cells according to ISO-10993-5 guidelines. Subsequently, transfection efficiency and cytotoxicity of BMP-7 cmRNA in hMSCs on THA-Col bulk and microgels was investigated by *metridia* luciferase (MetLuc) expression, BMP-7 protein secretion, LDH release, metabolic activity, and analysis of cell viability. Furthermore, in vitro osteogenic differentiation of hMSCs transfected with BMP-7 cmRNA on bulk or microgels was analyzed by alkaline phosphatase (ALP) activity, osteoprotegerin (OPG) secretion and gene expression of osteogenic markers.

2. Results

2.1. 3D Printed THA-Col Microgels

THA-Col microgels were prepared by first mixing reconstituted THA with Col, followed by the addition of crosslinkers HRP, Ru, and SPS (**Figure 1**). Hydrogen peroxide was then added to the mixture to initiate enzymatic crosslinking, while at the same time Col was neutralized using sodium hydroxide. After enzymatic crosslinking, a partially crosslinked hydrogel was formed that could be used either as a bulk material or further processed to make microgels. To produce microgels, the bulk THA-Col gel was passed three times through a grid of 100 μm mesh size, optimized based on previous work and preliminary tests.^[43] The THA-Col microgels were then ready to be used for 3D printing, followed by crosslinking using visible blue light for shape retention.

Rheological characterization was performed to assess the THA-Col microgels ink suitability for 3D printing.^[44] Here, the amplitude sweep revealed a similar G' value for both the bulk and the microgel samples, while G'' was higher for microgels compared to the bulk THA-Col (**Figure 2a**). The viscosity curve showed that THA-Col microgels exhibited viscoelastic and shear thinning behavior (**Figure 2b**). The elastic recovery of microgels subjected to alternating low (1%) and high (500%) strain displayed a decrease in G' under high strain, re-

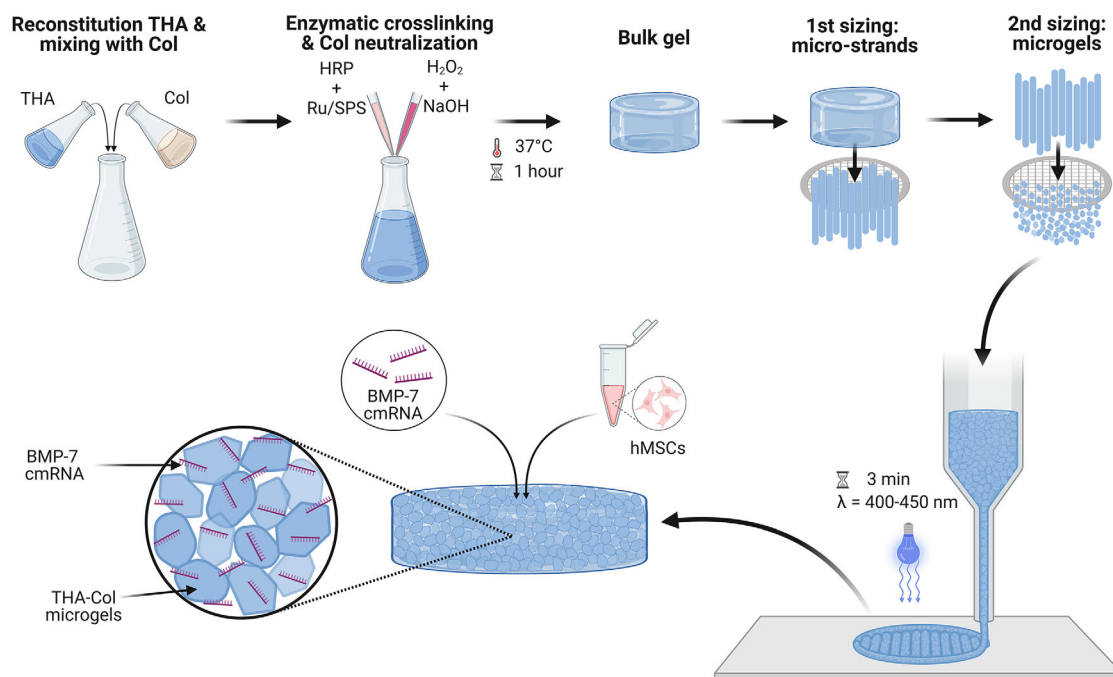


Figure 1. Schematic of THA-Col microgel matrix fabrication.

sulting in $G' < G''$. Upon returning to low strain, both G' and G'' were restored to values comparable to their initial measurements (Figure 2c).

Printability of THA-Col microgels was analyzed by filament spreading and uniformity, filament fusion, pore geometry, and overhanging pillar tests (Figure 2d,e). Quantification of filament spreading resulted in a ratio of 1.3, indicating that the diameter of the printed strut was larger than the 3D printing nozzle (Figure 2f). The measured filament uniformity was slightly above 1, indicating generally uniform filaments with minor aberrations (Figure 2g). The fused segment length (F_s) from the meandering pattern was determined to be ≈ 0.6 (Figure 2h). Pore geometry of the lattice exhibited a P_r value below 1, with some variation among replicas as illustrated by standard deviation, and some pores closer to a perfect square $P_r = 1$ (Figure 2i). The overhanging filament on the pillar structure showed capacity to bridge all the gaps up to 6 mm (Figure 2e).

2.2. THA-Col Bulk and Microgel Matrix Characterization

THA-Col bulk and microgel matrices were compared by swelling, degradation, and mechanical properties. Over 48 h, no swelling was observed for both bulk and microgels (Figure 3a). The degradation of THA-Col bulk and microgels in a solution containing 100 U mL⁻¹ hyaluronidase was assessed over a 48-h period. The results indicated that microgels exhibited a significantly higher degradation rate than the bulk THA-Col (Figure 3b). After 48 h, 50% of the microgels had degraded, whereas the bulk material showed only a 28% degradation rate. The unconfined compression test revealed a compressive modulus of 34 kPa for bulk THA-Col, whereas the microgels exhibited a significantly lower value of 27 kPa (Figure 3c).

2.3. Cytotoxicity of THA-Col Bulk and Microgels on L929 Cells

Cytotoxicity of THA-Col bulk and microgels was investigated according to the ISO-10993-5 guidelines (Figure 4a). Cells treated with conditioned medium from bulk and microgels showed no significant decrease in metabolic activity, DNA content, or LDH release when compared to the untreated cells in the negative control (Figure 4b–d, respectively). Similarly, cell viability according to Live/Dead staining showed comparable results between negative control and cells that were incubated with conditioned media from THA-Col bulk or microgels (Figure 4e).

2.4. In Vitro Transfection of hMSCs on THA-Col Bulk and Microgels with MetLuc and BMP-7 cmRNA

hMSC secretion of *metridia* luciferase and BMP-7 following cmRNA transfection were assessed after 1, 3, and 7 days (Figure 5a). hMSCs pre-transfected in suspension before seeding on THA-Col gels were used as a positive control. *Metridia* luciferase showed the highest activity at day 1, followed by day 3 and was lowest at day 7 for all groups (Figure 5b). At day 1, the highest activity was in the transfected groups, while no activity was measured in the non-transfected hMSCs on the THA-Col bulk and microgels. No significant differences were detectable between the transfected groups on the gels and the pre-transfected positive controls. The transfection with BMP-7 cmRNA resulted in similar trends (Figure 5c), with the highest amount of BMP-7 detected at day 1 and decreased concentrations at day 3 and day 7. At all timepoints, no BMP-7 was secreted by the non-transfected cells. A non-significant trend was observed, indicating that the positive control pre-transfected cells produced higher levels of BMP-7 when compared to the cells that were transfected on the matrices.

Legend

- Bulk
- Microgels

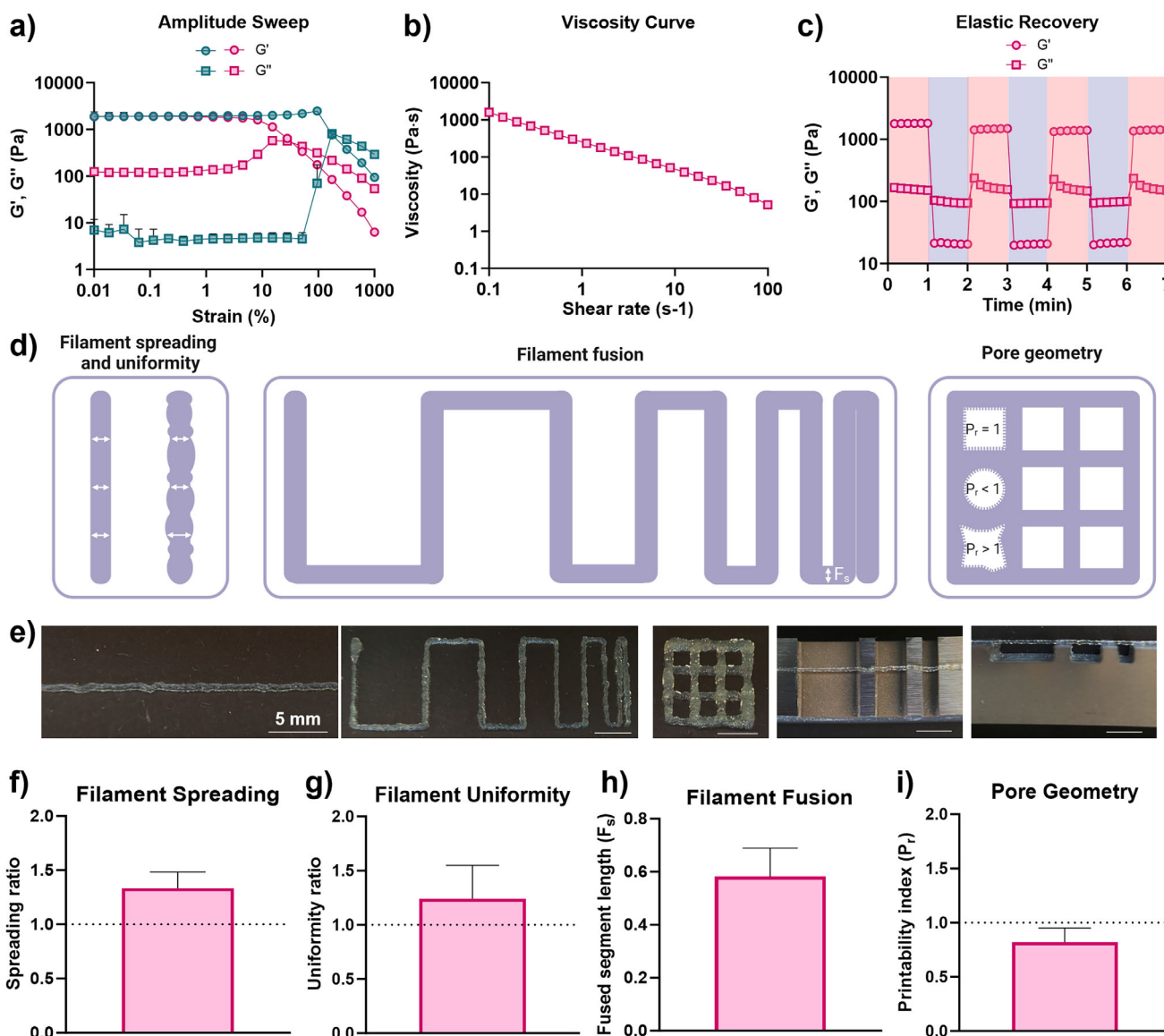


Figure 2. 3D Printed THA-Col microgels matrix. a) Amplitude sweep after enzymatic crosslinking, $n = 3$. b) Viscosity curve of microgels, $n = 3$. c) Elastic recovery of microgels with alternating low (1%) and high (500%) strain, $n = 3$. d) Schematic evaluation of printability assessment (filament spreading and uniformity, filament fusion, and pore geometry). e) 3D printed THA-Col microgels strands, filament fusion, lattice, and overhanging pillar, scale bar = 5 mm. Quantification of f) filament spreading, $n = 3$, g) filament uniformity, $n = 3$, h) filament fusion, $n = 3$, and i) pore geometry, $n = 3$. THA = Tyramine modified hyaluronic acid, Col = collagen.

No significant differences could be observed between the transfected hMSC on THA-Col bulk and microgels.

A non-significant trend in LDH release was also observed, with the highest levels measured at days 1 and 3 for the groups transfected with BMP-7, particularly peaking on day 1 in the pre-transfected groups (Figure 5d). By day 7, LDH levels across all groups were comparable. The metabolic activity of transfected hMSCs in both bulk and microgels was similar to that of the CCP negative control, although a slight increase was observed

in the microgel group (Figure 5e). All transfected groups exhibited significantly reduced metabolic activity. Additionally, cell viability assessed through Live/Dead staining corroborated these findings, showing a lower number of viable cells in the transfected groups (Figure 5f).

Cell morphology, qualitatively evaluated based on Calcein fluorescence, showed good adhesion for MSCs grown on both bulk and microgels (Figure S2a,b, Supporting Information, respectively).

Legend

- Bulk
- Microgels

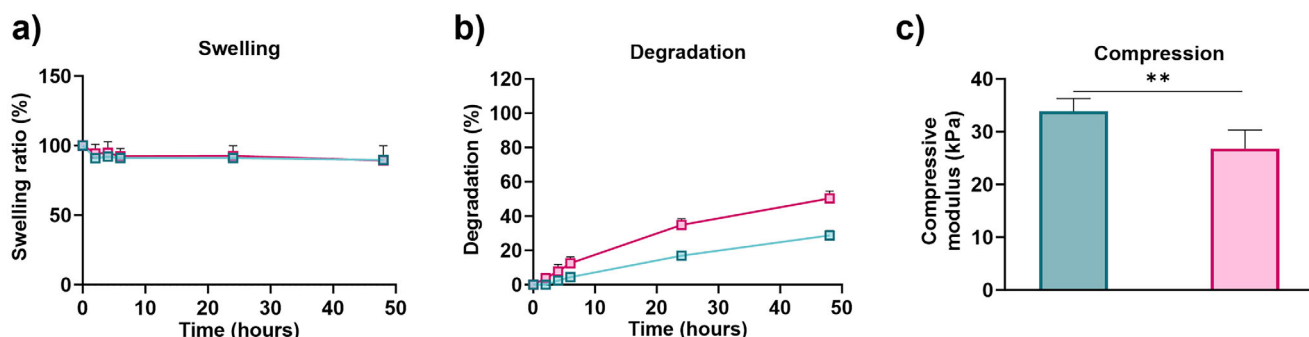


Figure 3. THA-Col bulk and microgel matrix characterization. a) Swelling ratio in PBS, $n = 6$. b) Degradation in 100 U mL⁻¹ hyaluronidase after 24 h of swelling, $n = 6$. c) Compressive modulus after 24 h of swelling, calculated between 0 and 10% strain, $n = 6$. One-way ANOVA with Šidák correction was used for statistical analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns = no significance. THA = Tyramine modified hyaluronic acid, Col = collagen.

2.5. Osteogenic Differentiation of hMSCs Transfected with BMP-7 cmRNA on THA-Col Bulk and Microgels

The ability of BMP-7 cmRNA transfected hMSCs to undergo osteogenic differentiation on THA-Col bulk and microgels was assessed over 28 days by ALP production, OPG secretion in the medium, and gene expression (Figure 6a).

Both ALP activity and DNA content were highest on the THA-Col microgels with non-transfected cells (Figure 6b). In this group ALP activity was significantly higher compared to the same group on bulk THA-Col gels. Interestingly, ALP activity of hMSCs transfected on the biomaterials or pre-transfected was comparable. Among the BMP-7 cmRNA transfected groups, similar values were observed, with a trend for slightly higher ALP activity for cells on THA-Col microgels compared to the bulk. DNA content was the highest in the non-transfected groups as expected, with a non-significant higher trend on the microgels (Figure 6c). In the BMP-7 cmRNA transfected groups, similar DNA content was measured, although a slight increase was observed for hMSCs cultured on microgels. ALP activity normalized to DNA was significantly higher in non-transfected hMSCs on microgels compared to the same group on bulk THA-Col (Figure 6d). For other groups, while no statistical significance was detected, trends suggested that the cells on bulk gels had slightly higher ALP/DNA in the pre-transfected group compared to the cells transfected on the gel. Conversely, this tendency was reversed for the hMSCs on the microgels. ALP activity results were corroborated by ALP staining (Figure 6e), with the most intense ALP staining on the non-transfected hMSCs on the microgels, followed by the BMP-7 cmRNA transfected cells on microgels. On the THA-Col bulk gels, very little to no staining could be observed.

OPG levels in the medium are reported in Figure 6f,g. Similarly to ALP activity, there were no differences in OPG/DNA between hMSCs pre-transfected or transfected on the materials. In general, for all groups an increasing trend over time was revealed, although at each timepoint no statistically significant differences were observed between groups, due to high variability.

For OPG/DNA at week 2 (Figure 6g), BMP-7 cmRNA transfected groups on either bulk or microgels showed increased values compared to the non-transfected control. Additionally, gene expression levels of several osteogenic markers were measured (Figure S3, Supporting Information). While most gene expression results showed no statistical differences between groups due to high standard deviations, the *RUNX2/SOX9* ratio demonstrated a trend that aligns with the observed ALP activity. Significant experimental variability is frequently noted when utilizing patient-derived cells. Nevertheless, primary cells hold greater clinical relevance, and similar variations in outcomes are also evident among human patients. Overall, our findings highlight a promising area for further investigation.

3. Discussion

The recent COVID-19 pandemic has catalyzed a swift evolution of RNA-based technologies, thereby creating numerous opportunities for their application in other sectors. cmRNAs are particularly interesting, as they hold enormous potential to overcome the shortcomings of protein delivery and gene (DNA) therapy.^[13] For exploiting this potential in bone tissue engineering, the cmRNA combination with suitable biomaterials allowing successful transfection is necessary.^[22] This study aimed to fill this gap developing and evaluating a 3D printable active matrix based on THA-Col microgel matrix to specifically deliver BMP-7 cmRNA and promote bone healing.

HA and Col are natural biopolymers present in the body's ECM, with Col serving as a primary constituent of natural bone. The integration of THA and Col harnesses the benefits of each individual component while mitigating their drawbacks. For instance, printing Col alone presents significant challenges due to its viscoelastic properties; however, when combined with THA, it becomes suitable for printing.^[3,29] Although 3D printing of bulk THA-Col is possible, microgels further improve 3D printing properties while also creating porosity to enhance cell proliferation and migration.^[30–32] In this study, bulk THA-Col was

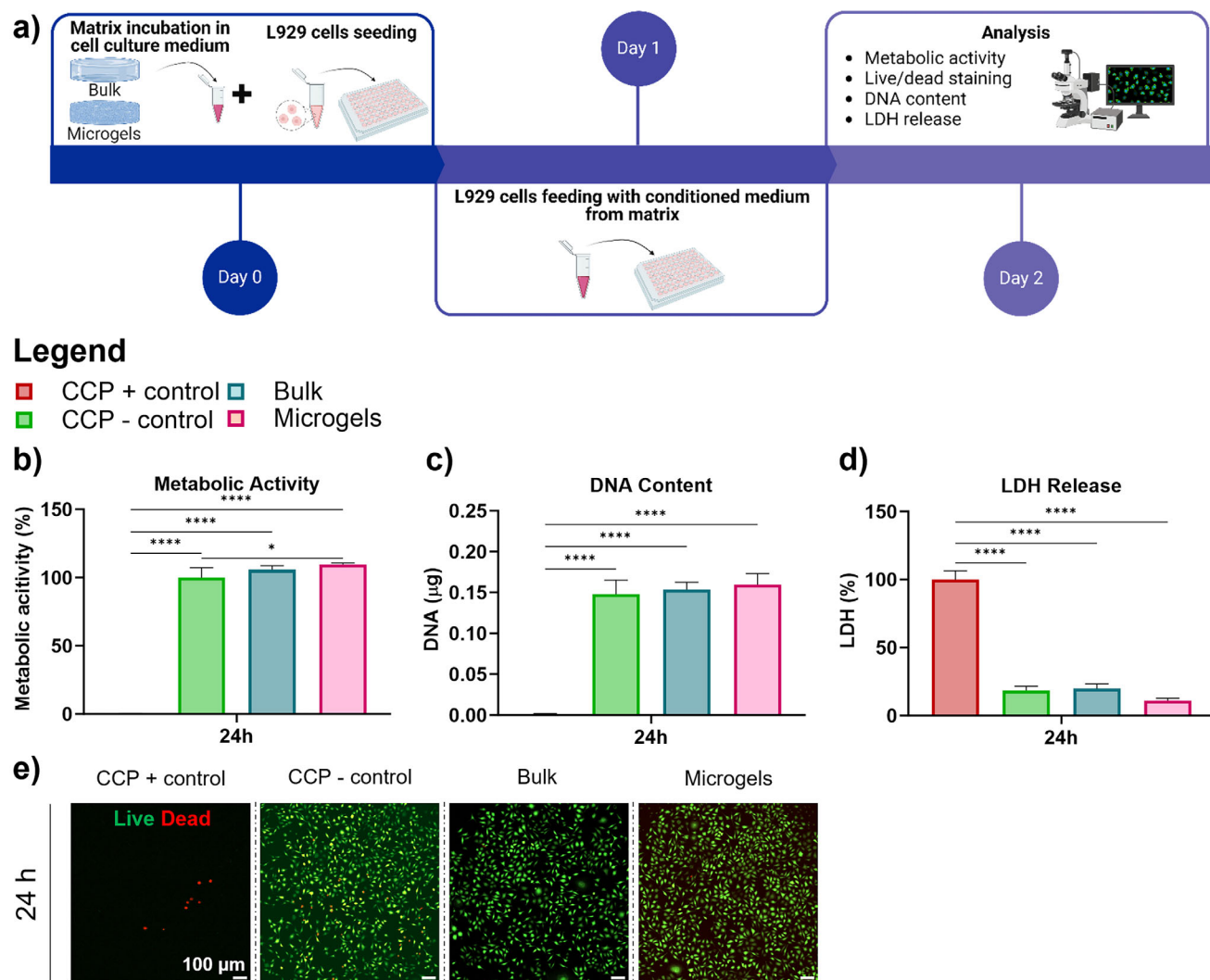


Figure 4. Indirect cytotoxicity of THA-Col bulk and microgel matrices tested with L929 cells. a) Schematic of in vitro indirect toxicity experimental set-up. b) Metabolic activity after 24 h normalized to CCP – control, $n = 4$. c) Quantification of DNA content after 24 h, $n = 4$. d) Quantification of LDH release after 24 h, $n = 4$. e) Representative images of live (green) and dead (red) staining after 24 h, scale bar = 100 μm, $n = 4$. One-way ANOVA with Šidák correction was used for statistical analysis, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns = no significance. CCP = Cell culture plastic, CCP + control = Cell culture plastic treated with 0.1% Triton, CCP – control = Cell culture plastic with unconditioned medium, THA = Tyramine modified hyaluronic acid, Col = collagen, LDH = Lactate dehydrogenase.

mechanically fragmented with a simple, reproducible and scalable process. The resulting microgels exhibited an excellent viscoelastic profile with elastic shear recovery properties ideal for 3D printing purposes, with improvements compared to the bulk formulation^[3] and confirming previous reports.^[41]

The synergy between THA and Col extends to the swelling properties. THA is hydrophilic and may display excessive swelling, while Col is known for its cell-mediated shrinking. Their combination was previously shown to give rise to a composite bulk gel with moderate swelling during 28 days in cell culture.^[3] The microgels in the present study confirmed these results, and no swelling or shrinking was detected for both the THA-Col bulk and microgels. However, the microgels did degrade faster compared to the corresponding bulk. This might be attributed to the higher porosity and surface area exposed

to the hyaluronidase. This higher porosity and relative movement of the microgel particles may also be responsible for the lower compressive modulus compared to a bulk gel, which has also been observed in another study using HA-based granular hydrogels.^[45] Although the constructs remained cohesive during in vitro culture and degradation tests, caution is necessary when extrapolating these results to in vivo conditions. Indeed, several other mechanisms influencing degradation occur in vivo, including blood and lymphatic circulation, oxidative stress, and active degradation by other cell populations (e.g., immune cells) which cannot be reproduced in vitro.

Although there is substantial evidence supporting the effectiveness of BMP-2 and BMP-7-based clinical products, concerns persist regarding the adverse effects linked to the use of supraphysiological concentrations.^[4,7,8] BMP-7 was one of the first

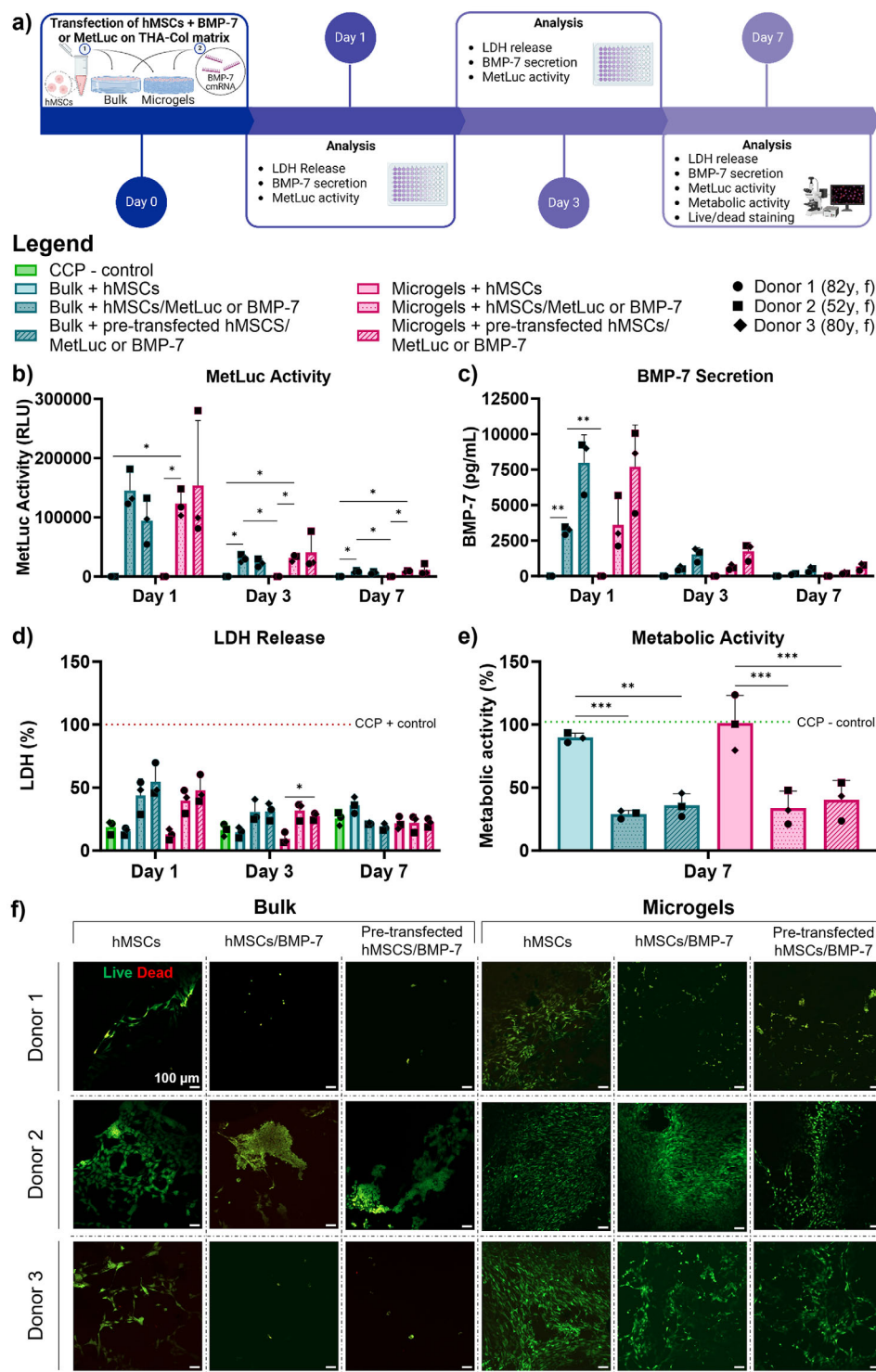


Figure 5. Transfection efficiency and cytotoxicity of BMP-7 cmRNA with hMSCs on THA-Col bulk and microgels. a) Schematic of in vitro transfection efficiency and cytotoxicity set-up. b) Metridia luciferase expression after 1, 3, and 7 days, $n = 3$. c) BMP-7 secretion in culture medium after 1, 3, and 7 days, $n = 3$. d) Quantification of LDH release after 1, 3, and 7 days, $n = 3$. e) Metabolic activity after 7 days normalized to CCP - control, $n = 3$. f) Representative images of live (green) and dead (red) staining after 7 days, scalebar = 100 μm , $n = 3$. One-way ANOVA with Šidák correction was used for statistical analysis in figure c and two-way ANOVA with Tukey's multiple comparison test was used for figure b,e,f, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns = no significance. THA = Tyramine modified hyaluronic acid, Col = collagen, MetLuc = Metridia luciferase.

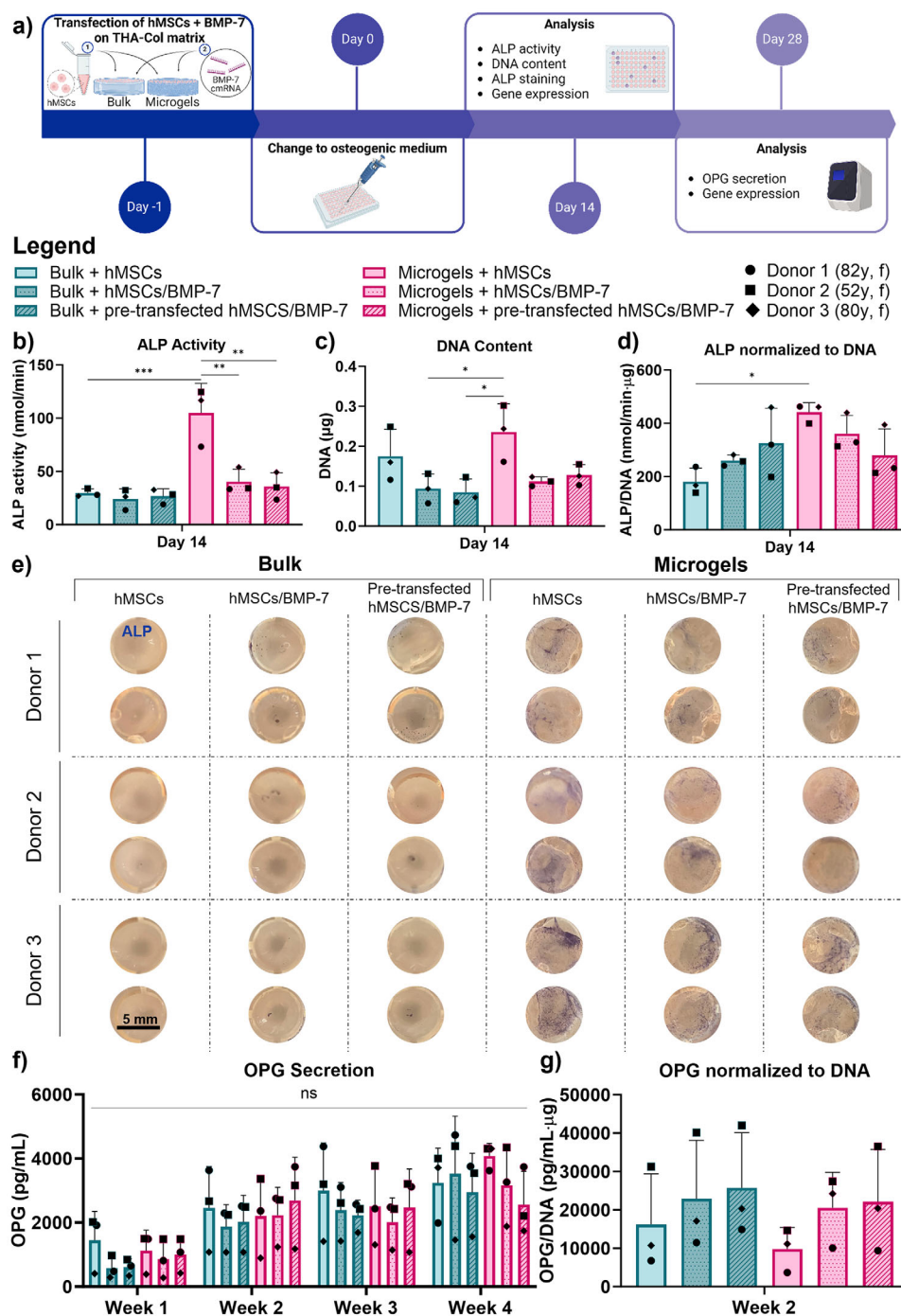


Figure 6. Osteogenic differentiation of hMSCs on THA-Col bulk and microgels matrices combined with BMP-7 cmRNA. a) Schematic of in vitro osteogenic differentiation experimental set-up. b) ALP activity after 14 days, $n = 3$. c) Quantification of DNA content after 14 days, $n = 3$. d) ALP activity normalized to DNA content after 14 days, $n = 3$. e) Images of ALP staining (blue) after 14 days, scalebar = 5 mm, $n = 3$. f) OPG secretion in culture medium pooled together per week, $n = 3$. g) OPG secretion at week 2 normalized to DNA content after 14 days, $n = 3$. One-way ANOVA with Šidák correction was used for statistical analysis in figure b,c,d and two-way ANOVA with Tukey's multiple comparison test was used for figure f, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns = no significance. THA = Tyramine modified hyaluronic acid, Col = collagen, ALP = Alkaline phosphatase, AR = Alizarin Red, OPG = Osteoprotegerin.

orthobiologics used in the clinics, however the corresponding product (OP-1) was discontinued for both market reasons and for concerns about side effects,^[46] which were due to the administration of a massive dose without a controlled release mechanism. RNA technology opens new opportunities for the use of BMP-7, with growing interest due to increasing number of reports of side effects for BMP-2.^[8] Research aimed at overcoming these limitations by using mRNA encoding the desired growth factor, similar to SARS-CoV-2 vaccines is highly promising. Specifically, chemical modification enhances mRNA stability and reduces its immunogenicity.^[11–13]

Key steps in developing cmRNA-based active matrices for bone regeneration include preservation of transfection efficiency, of the growth factor's biological activity, and of the transfected cells' viability. In this study, transfection efficiency was tested with *metridia* luciferase as reporter, with BMP-7 secretion, and with osteogenic differentiation of hMSCs. Here, the key result is that hMSCs transfected on the biomaterials showed levels comparable to pre-transfected hMSCs, based on both ALP/DNA activity, and on OPG secretion. This indicates that both granular and bulk THA/Col gels can be used as activated matrices to deliver BMP-7 cmRNA with preservation of biological activity. Considering the results of the printability, cytocompatibility, and metabolic activity after transfection, the granular version of the gel emerges as lead composition.

hMSCs were successfully transfected on both bulk and microgels, with highest levels at day 1, as expected. The different protein expression levels for *metridia* luciferase and BMP-7 secretion may be attributed to the different detection methods, as well as to intrinsic properties such as the different size, isoelectric point, and biological function of the 2 proteins. The transfection with BMP-7 cmRNA lead to a non-significant increase in LDH release at day 1 and day 3, while levels recovered to values similar to non-transfected hMSCs at day 7.

At day 7, transfected hMSCs showed a decline in metabolic activity and a reduced number of viable cells. Together, these observations indicate that transfection initially led to a degree of cytotoxicity. Although there was a gradual recovery over the course of the week, the total cell count remained lower, leading to diminished metabolic activity and fewer live cells at this time point. Research has shown that cmRNA transfection may result in decreased cell viability,^[12] which could be a direct consequence of the transfection process or of the initial burst release that follows successful transfection. One possible strategy to refine the control over the release profile would be optimizing BMP-7 cmRNA dose, or embedding it into microparticles made THA-Col or another microgel, allowing for its gradual release as the hydrogel degrades. Future research could focus on determining an appropriate matrix for cmRNA bone embedding to enhance the regulation of release kinetics. One limitation of our study is the lack of control groups where hMSCs were treated with either a scrambled non-coding cmRNA sequence or with a lipid vector. For our work we have selected a first-generation lipid-based nanodelivery vehicle, which is very well known in the field, and therefore more suitable to assess the developed biomaterials as substrates for direct transfection. Cationic lipids have a long track record of use as RNA delivery systems, and their toxicity is well documented.^[47] Future developments for the clinical translation of the present work will consider the use of other delivery

vehicles with decreased toxicity. Concerning the optimization of the cmRNA transfection protocol, we relied on previous work.^[16,19,48,49]

Previous studies investigated the stability of cmRNA compared with the parent mRNA sequence. Unmodified mRNA is known to elicit a strong activation of the innate immune system which accelerates decay of unmodified versus modified mRNA in vivo.^[50,51] These degradation mechanisms mediated by innate immunity are difficult to capture in off-the-shelf stability studies or with cells in culture. Kormann et al. found that chemically modified mRNA is much more stable in mouse lungs, showing 3- to 6-fold higher levels than unmodified mRNA between 8 h and 7 days post-administration.^[11] De La Vega et al. documented varying decay kinetics of bioluminescence signals after administering chemically modified firefly luciferase-encoding mRNA via lipid nanoparticles in a rat model of femoral bone defects. A notable bioluminescence signal was still detected 27 h post-administration.^[17] The efficacy of delivery systems is vital for the in vivo stability of these modified mRNAs, and the field has seen significant advancements.^[50]

In vitro, osteogenic differentiation of non-transfected hMSCs on THA-Col showed significantly higher ALP activity on microgels compared to bulk, which was also confirmed with a more intense ALP staining on microgels compared to bulk gels. hMSCs transfected on microgels showed reduced ALP activity and staining compared to non-transfected cells. A non-significant trend with lower DNA content was also observed in transfected cells, in agreement with previous LDH release, metabolic activity, and Live/Dead staining results. Independently of transfection, hMSCs on bulk THA-Col showed similar ALP activity, DNA content, and ALP staining. ALP plays an essential role in the process of new bone mineralization,^[52] therefore it is widely used as distinctive marker of osteogenic differentiation. Considering that ALP undergoes inherent fluctuation over time during osteogenic differentiation, and considering the intrinsic variability in biological response of cells from different donors, our experiments can not distinguish between a general dampening or a temporal shift in the profile of ALP activity. Cell differentiation was also analyzed by examining the gene expression levels of osteogenic markers. *RUNX2/SOX9* ratio at day 14 correlated with ALP activity, with higher levels in untransfected cells on microgels. However, other genes did not show any clear trend. These limitations were mitigated by evaluating other markers, such as OPG. This soluble protein functions as a decoy receptor that binds to receptor activator of nuclear factor κ B ligand (RANKL), where it inhibits osteoclast differentiation and it plays an important role in bone density regulation.^[53,54] In previous in vitro osteogenesis studies, a clear difference between hMSCs in basal medium or osteogenic differentiation medium was observed, indicating OPG as a predictive marker.^[3,53] In fact, groups that were transfected with BMP-7 cmRNA show higher OPG/DNA compared to the control groups. Additionally, OPG/DNA confirmed ALP/DNA results indicating successful transfection on the biomaterials.

A limitation of this study is the lack of quantification of direct mineralization to assess osteogenesis. Alizarin Red staining after 28 days of osteogenic differentiation was performed, however it was not possible to reliably distinguish the mineral deposition by differentiated hMSCs from the unspecific background staining from the THA-Col matrix (data not shown). False pos-

itive staining of unincubated matrices was previously observed in other studies using, for example, commercially available Col membranes.^[55]

Bones can heal via two different routes, intramembranous or endochondral ossification. While BMPs have been reported to induce endochondral ossification in vivo, the in vitro model used in this study follows a direct mineralization process.^[56,57] Therefore, caution should be used when transferring our results to healing long or flat bones in vivo.

For our experiments, primary bone marrow human donor-derived hMSCs were used. The considerable variability observed among donors is anticipated and common when utilizing primary cells. Although standard statistical analysis may not indicate significant trends, the findings remain biologically relevant. On the other side, the use of primary patient-derived cells significantly adds to the clinical relevance of our results.

Building on previous advancements in cmRNA technology, our work introduces a 3D printable biomaterial allowing BMP-7 cmRNA transfection preserving the same level of biological activity of pre-transfection on a dish, with the difference that our approach could be directly applied in a minimally invasive manner, avoiding 2 surgeries (tissue harvesting and re-implantation) and autologous cell transfection under GMP conditions. Future efforts will focus on minimizing the cytotoxicity effects caused by either a rapid release of the mRNA or the selection of the transfection vehicle. Additionally, we aim to further enhance osteogenic differentiation, which in this study may have been hindered by cell loss resulting from this toxicity. Thus, combining an alternative approach to mRNA encapsulation and the use of osteoinductive calcium phosphate particles,^[3,12,58] for instance, can work synergistically to improve the bone healing properties of the THA-Col microgels that deliver BMP7 cmRNA.

4. Conclusion

This study introduces 3D printable THA-Col microgels as activated matrices to transfect hMSCs with BMP-7 cmRNA for promoting osteogenic differentiation. THA-Col microgels showed higher cell viability compared to bulk gels, supported transfection with BMP-7 cmRNA and supported osteogenic differentiation of hMSCs in vitro. This work confirms the potential and usability of cmRNA technology in bone regeneration, and it contributes to moving the development of patient-specific bone graft substitutes, toward offering an alternative to bone autografting.

5. Experimental Section

THA Synthesis: The synthesis of THA was performed using a previously described method.^[27] In short, hyaluronic acid (HA, 280–290 kDa, 5 mM carboxylic groups; Contipro Nutrihy) was functionalized using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, TCI Europe) amidation together with tyramine HCl (Roth) by blending at a stoichiometric ratio of 1:1:1 for 24 h at 37 °C. Following, THA was precipitated by dropwise addition of ethanol (96% v/v) into the solution. THA was filtered using a Gooch filter no. 2, and finally the powder was dried under vacuum. The degree of substitution of THA was ≈6%, measured by absorbance reading at 275 nm (Infinite® 200 PRO microplate reader, TECAN) against a calibration curve of tyramine HCl. Proton nuclear magnetic resonance (NMR) spectrum was acquired on a 300 MHz

Bruker Avance III using deuterium oxide as a solvent; the graphical layout of the spectrum was produced with NMRium (Figure S1, Supporting Information).

THA-Col Bulk and Microgels Preparation: The THA-Col hydrogel was prepared by reconstituting THA to a final concentration of 17.5 mg mL⁻¹ in phosphate-buffered saline (PBS) at 4 °C under rotation. Dissolved THA was then heat sterilized for 20 min at 121 °C. Sterilized THA was mixed with 0.1 U mL⁻¹ horseradish peroxidase (HRP), 0.37 mM Ruthenium tris(2,2-bipyridyl)dichlororuthenium(II) hexahydrate (Ru), 5 mM Sodium Persulfate (SPS), and Col at a final concentration of 2.5 mg mL⁻¹ (rat tail collagen type I in 0.2 N acetic acid, Corning). The THA-Col liquid mixture was then neutralized by the addition of 6 mM sodium hydroxide (NaOH, Roth) and enzymatically crosslinked by addition of 0.17 mM hydrogen peroxide (H₂O₂, Sigma-Aldrich). Subsequently, for bulk gels, 100 µL of the hydrogel solution was dispensed into 96-well plates, while for microgels, the solution was retained in a syringe. In both cases, the samples were allowed to crosslink by incubating for 60 min at 37 °C. To prepare microgels, enzymatically crosslinked THA-Col was passed three times through a cell strainer with a membrane pore size of 100 µm (Falcon) according to a previously described method.^[41] Microgels were then added to 96-well plate, with a volume of 100 µL per well. Bulk and microgels were fully crosslinked by light exposure (456 nm) for 3 min (LED IP FL-30 SMD blue, 30W, 100–240 V AC, 50/60 Hz, EuroLit).

3D Printing of THA-Col Microgels: For 3D printing, non-light crosslinked microgels were transferred into 3 CC cartridges (Nordson). The ink was then 3D printed with an extrusion-based 3D printer (3D Discovery™, RegenHU) using cylindrical needles of 22 G with an inner diameter of 0.41 mm (Nordson EFD), with pressure set at 1.6 bar, and writing speed of 4 mm s⁻¹ with subsequent light crosslinking (456 nm) for 3 min (LED IP FL-30 SMD blue, 30W, 100–240 V AC, 50/60 Hz, EuroLit). Printability was assessed by filament spreading and uniformity, filament fusion, pore geometry, and filament overhanging a pillar.^[59]

Filament spreading and uniformity was evaluated by 3D printing a single filament with a length of 60 mm. Filament spreading ratio was then assessed using ImageJ by measuring the filament diameter at three distinct locations (¼, ½, and ¾ of the filament length) of the extruded strand and normalizing to the inner needle diameter. Filament uniformity ratio was measured using ImageJ by manually outlining the extruded strand on the top and bottom and measuring the length. This value was normalized by the length of a perfectly straight line. A value of 1.0 represents maximum uniformity, while higher values indicate less uniformity.^[60]

Filament fusion was evaluated by 3D printing parallel filaments in a meandering pattern with gradually decreasing filament spacing (7, 5, 4, 3, 2, 1, and 0 mm). Fused segment length ($\bar{f}_s$) was then measured using ImageJ for the smallest possible filament spacing that did not lead to filament fusion.

The pore geometry was analyzed by extruding a 1-layer grid structure of 9 × 9 × 0.41 mm (length × width × height), where the 3D printed pores were evaluated for their shape by measuring the printability index (P_r). P_r values were calculated using the formula: $P_r = (L^2) / (16 \times A)$. Here, L represented the internal perimeter and A indicated the internal area of each pore.^[61] P_r value of 1 would suggest a perfectly square shape, while a value of $P_r < 1$ would indicate overly circular pores, and $P_r > 1$ values would point to irregular pore shapes.

For the filament overhanging, a single filament was 3D printed on top of a pillar structure with increase gap size (1, 2, and 6 mm).

THA-Col Bulk and Microgels Matrix Characterization—Rheological Characterization: Rheological characterization was performed using an Anton Paar MCR-302 rheometer (Anton Paar GmbH) equipped with a Peltier temperature control device. All tests on the THA bulk or microgels samples ($n = 3$) were performed using a plate-plate geometry (PP-25 Probe, 25 mm diameter), with a gap size of 0.1 mm for bulk and 1.1 mm for microgels, and a 0.1 N normal force. All the measurements were conducted at 20 °C, and silicone oil (Sigma-Aldrich) was applied to the external border to prevent drying of the gel during the measurement.

The oscillatory strain sweep was performed using a strain between 0.01% to 1000%, with 1 Hz angular frequency.

The viscosity was assessed using a shear rate between 0.01 to 100 1/s to evaluate the viscoelastic and shear thinning properties of the THA-Col microgels.

The elastic recovery of the THA-Col microgels was analyzed by exposing them to cycles of alternating low (1% strain) and high (500% strain), with 1 Hz angular frequency.

THA-Col Bulk and Microgels Matrix Characterization—Swelling: Non-freeze-dried THA-Col bulk and microgels assessed for swelling. Either bulk or microgels were cast in molds of polydimethylsiloxane (PDMS) with a diameter of 6 mm and a thickness of 3 mm ($n = 6$). Bulk and microgels were transferred into PBS solution to allow for swelling at 37 °C. Using an electronic balance, bulk or microgels were weighed at appointed times (0, 2, 4, 6, 24, and 48 h). The swelling ratio of bulk and microgels at given time points was determined using the following formula: Swelling ratio (%) = $(\text{Weight } t_x / \text{weight } t_0) \times 100$.

THA-Col Bulk and Microgels Matrix Characterization—Degradation: Bulk and microgels were prepared in PDMS molds with a diameter of 6 mm and a thickness of 3 mm ($n = 6$ each) and allowed for swelling in PBS for 24 h at 37 °C. Both bulk and microgels were then transferred to 100 U mL⁻¹ hyaluronidase in PBS solution and incubated at 37 °C for degradation. Samples were weighed at appointed times (0, 2, 4, 6, 24, and 48 h) using an electronic balance. The degradation of bulk and microgels at the different time points was calculated using the following formula: degradation (%) = $1 - (\text{Weight } t_x / \text{weight } t_0) \times 100$.

THA-Col Bulk and Microgels Matrix Characterization—Compression: Bulk and microgels were casted in a PDMS mold with a diameter of 6 mm and a thickness of 3 mm ($n = 6$ each) and were incubated in PBS for 24 h at 37 °C to allow any swelling prior to mechanical testing using an LTM 1 (ZwickRoell). Bulk and microgels were tested under unconfined uniaxial compression using preload of 0.25 N and speed of 1%/s. The compressive modulus was calculated from the stress-strain curve as the slope of the linear region between 0 and 10% strain.

Cell Culture—L929 Cells: L929 mouse fibroblasts cells (Sigma-Aldrich) were cultured in basal medium consisting of low glucose (1 g L⁻¹) Dulbecco's Modified Eagle Medium (LG-DMEM, Gibco) supplemented with 10% v/v fetal bovine serum (FBS, Corning), and 1% v/v Pen-Strep (final concentration 100 U mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin, Gibco) at 37 °C in an atmosphere of 5% CO₂ and 90% humidity. Cell culture medium was changed every second day. For all cell culture experiments involving L929 cells, cells at passage 19 were used.

Cell Culture—Human Mesenchymal Stromal Cells Isolation and Expansion: Bone marrow aspirates from three female donors between the age of 52 and 82 years were used to isolate hMSCs, as described in a previous study.^[62] The aspirates were obtained from patients undergoing orthopedic surgeries at the Inselspital Bern (Clarification of Responsibility Req-2023-00198). The Swiss Human Research Act does not apply to research involving anonymized biological material and/or anonymously collected or anonymized health-related data. General Consent, which also covered anonymization of health-related data and biological material, was obtained from all donors.

After isolation hMSCs were frozen down at passage 1 and when thawed expanded in Minimal Essential Media alpha modification (α-MEM, Gibco) supplemented with 10% v/v FBS (Corning), 1% v/v Pen-Strep (100 U mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin, Gibco) and 5 ng mL⁻¹ basic fibroblast growth factor (bFGF, Fitzgerald Industries International) at 37 °C in an atmosphere with 5% CO₂ and 90% humidity. The medium was changed every second day. hMSCs at passage 3 were used for all experiments.

Cell Culture—In Vitro Indirect Toxicity: Indirect toxicity studies were performed following the ISO-10993-5 guidelines.^[63] 100 µL of THA-Col bulk and microgels in 96-well plates were incubated with 200 µL of basal medium for 24 h at 37 °C. Medium alone, without and THA-Col present, was incubated under identical conditions to serve as a negative control and medium supplemented with 0.1% Triton X-100 (Sigma-Aldrich) as positive control of cell death. L929 cells were seeded in 96-well plates at a cell seeding density of 10 000 cells cm⁻² and were left 24 h to attach ($n = 4$). After 24 h of incubating THA-Col bulk and microgels with medium, this conditioned medium was extracted from the gels and used to change the

medium of the attached L929 cells. L929 cells were cultured with the conditioned medium for a further 24 h and then assessed for cell proliferation and viability.

Quantification of Metabolic Activity: L929 cells metabolic activity (CellTiter-Blue®, Promega) was assessed according to the manufacturer's instructions. In short, cells were incubated for 2 h at 37 °C and 5% CO₂ with a 1:5 dilution of CellTiter-Blue® in basal medium. The fluorescence of the CellTiter-Blue® reagent in the medium was assessed using a microplate reader (Infinite® 200 PRO microplate reader, TECAN), with an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Quantification of DNA Content: L929 cells were lysed using 0.1% v/v Triton X-100 (Sigma-Aldrich) in 10 mM TrisHCl. DNA content was then assessed in duplicates using a DNA quantification assay (CyQUANT™ Assay, ThermoFisher) following the manufacturer's instructions. Fluorescence was measured using a microplate reader (Infinite® 200 PRO microplate reader, TECAN) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Live/Dead Staining: L929 cell viability was evaluated with a Live/Dead staining. After washing one time with PBS, cells were incubated in LG-DMEM containing 1 µM calcein-AM (Sigma-Aldrich) and 1 µM ethidium homodimer-1 (Sigma-Aldrich) for 30 min at 37 °C. After further washing, cells were maintained in LG-DMEM for confocal imaging (LSM800, Carl Zeiss). Dead cells, visible in red, were stained with ethidium homodimer-1 ($\lambda_{\text{ex}} = 561$ nm), while the cytoplasm of living cells, visible in green, was stained with calcein AM ($\lambda_{\text{ex}} = 488$ nm).

Quantification of LDH Release: Conditioned medium from L929 cells was collected used for lactate dehydrogenase (LDH) (Cytotoxicity Detection Kit^{PLUS}, Roche) measurements following the manufacturer's instructions. Absorbance values of samples in duplicates were measured at 490 nm using a plate reader (Infinite® 200 PRO microplate reader, TECAN). The percentage of LDH release was calculated by normalizing absorbance values from THA-Col bulk or microgel samples to the positive control.

Cell Culture—In Vitro Transfection of hMSCs with a Combination of THA-Col Bulk or Microgels and BMP-7 cmRNA: THA-Col bulk and microgels were prepared as described before with 100 µL gel per well in a 96-well plate. BMP-7 cmRNA lipoplexes were formed by mixing 1 µg µL⁻¹ cmRNA solution (Ethris GmbH) and Lipofectamine MessengerMAX (Invitrogen) in ratio 1:1 and incubating for 20 min at room temperature. Either bulk or microgels were then seeded with 50 000 hMSCs/gel ($n = 3$ donors, with each two replicates) and left for 30 min in the incubator. Next, for transfection, 1.56 pg BMP-7 or MetLuc cmRNA/cell was added. After 30 min in the incubator, transfection medium consisting of Opti-MEM (Gibco), 2% v/v FBS (Corning), and 1% v/v Pen-Strep (100 U mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin, Gibco) was added to reach a final volume of 200 µL. As a positive control hMSCs were pre-transfected (50 000 cells per gel with 1.56 pg cmRNA/cell) in suspension for 20 min at room temperature and subsequently were added to the gels. After 1 h in the incubator transfection medium was added to reach a volume of 200 µL. As a negative (untransfected) control, only hMSCs (50 000 cells per gel) were added without cmRNA. In this study, the following groups were assessed for each THA-Col bulk and microgels: negative control, positive control, and hMSCs transfected on the gel by MetLuc activity (for MetLuc cmRNA), cytotoxicity, BMP-7 production (for BMP-7 cmRNA), and osteogenic differentiation.

MetLuc Activity: The supernatants from cells transfected with MetLuc cmRNA, containing secreted MetLuc, was collected on days 1, 3, and 7 after transfection, and stored at -80 °C for subsequent analysis. To assess metridia luciferase activity, 50 µL of the supernatant was added to 50 µL of native coelenterazine (50 µM in degassed sodium phosphate buffer, pH 7.0; Synchem), as a substrate and following standard protocols.^[64] Luminescence was immediately measured at 480 nm at room temperature using a CLARIOSTAR plate reader (BMG Labtech).

Cytotoxicity of cmRNA Transfection: To assess the cytocompatibility of the BMP-7 cmRNA-activated matrices on hMSCs, metabolic activity at day 7,

Table 1. Sequences of primer and probe (forward, reverse, and probe sequence) and assay IDs of TaqMan Gene Expression Assays (Applied biosystem Assay ID) for gene expression analysis. Probes for *RPLP0*, *RUNX2*, *MMP13*, and *COL1A1* are modified with FAM at 5' and TAMRA at 3'. Probes in TaqMan Gene Expression Assays are conjugated with FAM at 5' and NFQ-MGB at 3'.

	Gene	Forward primer sequence	Reverse primer sequence	Probe sequence	Assay ID
Primer and probe	<i>RPLP0</i>	5'-TGG GCA AGA ACA CCA TGA TG-3'	5'-CGG ATA TGA GGC AGC AGT TTC-3'	5'-AGG GCA CCT GGA AAA CAA CCC AGC-3'	–
	<i>RUNX2</i>	5'-AGC AAG GTT CAA CGA TCT GAG AT-3'	5'-TTT GTG AAG ACG GTT ATG GTC AA-3'	5'-TGA AAC TCT TGC CTC GTC CAC TCC G-3'	–
	<i>MMP13</i>	5'-CGG CCA CTC CTT AGG TCT TG-3'	5'-TTT TGC CGG TGT AGG TGT AGA TAG-3'	5'-CTC CAA GGA CCC TGG AGC ACT CAT GT-3'	–
	<i>COL1A1</i>	5'-CCC TGG AAA GAA TGG AGA TGA T-3'	5'-ACT GAA ACC TCT GTG TCC CTT CA-3'	5'-CGG GCA ATC CTC GAG CAC CCT -3'	–
TaqMan Gene Expression Assays	<i>SOX9</i>	–	–	–	Hs00165814_m1
	<i>ALPL</i>	–	–	–	Hs00758162_m1
	<i>IBSP</i>	–	–	–	Hs00173720_m1
	<i>SPP1</i>	–	–	–	Hs00959010_m1
	<i>SPARC</i>	–	–	–	Hs00234160_m1
	<i>DLX5</i>	–	–	–	Hs00193291_m1

LDH release at day 1, 3, and 7, and cell viability were assessed according to the protocols described in paragraphs 2.5.3.1, 2.5.3.3, and 2.5.3.4. Quantification of BMP-7 Production: The secretion of BMP-7 by hMSCs transfected with BMP7 cmRNA on THA-Col bulk and microgels at day 1, 3, and 7 was determined via ELISA (R&D Systems) according to the manufacturer's protocol.

Cell Culture—In Vitro Osteogenic Differentiation: In vitro osteogenic differentiation was performed on THA-Col bulk and microgels as described in paragraph 2.5.4 ($n = 3$ donors, with each two replicates). After 24 h of incubation, the transfection medium was changed to osteogenic medium consisting of Opti-MEM (Gibco), 2% v/v FBS (Corning), 1% v/v Pen-Strep (100 U mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin, Gibco), 50 µg mL⁻¹ L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AA2P, Sigma), and 10 mM beta-glycerol phosphate (BGP, Sigma). The osteogenic medium in this study did not contain dexamethasone. The medium was changed twice a week.

ALP Staining, Quantification of ALP Activity, and DNA Content: Alkaline phosphatase (ALP) activity was analyzed at 14 days after cmRNA transfection and culture under osteogenic conditions. hMSCs on THA-Col gels were fixed using 70% ice-cold methanol for 15 min at 4 °C and were stained for ALP activity (Leukocyte Alkaline Phosphatase Kit, Sigma-Aldrich) using naphthol AS-MX phosphate and fast blue RR salt (Sigma-Aldrich) following the manufacturer's instructions.

ALP activity was also assessed in cell lysates. First, cells were lysed with 0.1% v/v Triton X-100 (Sigma-Aldrich) in 10 mM TrisHCl. ALP activity was then quantified by adding alkaline buffer solution, substrate solution (25 mg mL⁻¹ phosphate substrate in 1 mM diethanolamine buffer containing 0.5 mM MgCl₂, pH 9.8, Sigma-Aldrich) and deionised water to the cell lysate, followed by incubation at 37 °C for 15 min. After 15 min, the reaction was stopped by the addition of 0.1 M NaOH solution and absorbance values of the samples and ALP standard curve were read at 405 nm using a plate reader (Infinite® 200 PRO microplate reader, TECAN). The ALP activity was normalized to DNA content, which was measured according to the method described in paragraph "Quantification of OPG Production."

Quantification of OPG Production: Osteoprotegerin (OPG) secretion was analyzed by ELISA (R&D Systems) following the manufacturer's protocol. OPG was measured in the conditioned medium that was collected at every medium change during the 28 days of osteogenic differentiation, and samples were pooled together per week.

RNA Isolation and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR): RNA isolation was performed on samples collected

at days 0, 14, and 28 using TriReagent® (Molecular Research Center Inc.) following the manufacturer's protocol. The isolated RNA quantity and quality was measured using a UV-Vis spectrophotometer (NanoDrop One, Thermo Fisher). 0.25 µg of total RNA was used to synthesize cDNA with the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions on a Mastercycler Gradient thermocycler (Eppendorf). To perform RT-qPCR, 10 µl of reaction mixture containing 5 µl TaqMan Gene Expression Master Mix (Thermo Fisher), primer and probes (final concentration forward and reverse primer: 900 nM; final concentration TaqMan probe: 250 nM), diethylpyrocarbonate (DEPC) water, and cDNA (5 ng) was loaded per well in 384-well plates. A standard PCR program was used consisting of 2 min of initial heating to 50 °C, 10 min at 95 °C for polymerase activation and 40 cycles of alternating 95 °C for 15 s and 60 °C for 1 min for denaturation and annealing. Relative gene expression was calculated in duplicates of each donor applying the 2^{-ΔC_q} method with *RPLP0* as a reference gene and normalization to day 0 hMSCs before seeding. The expression of the following genes was evaluated: *RUNX2*, *SOX9*, *ALPL*, *IBSP*, *SPP1*, *MMP13*, *COL1A1*. The assay IDs of the TaqMan Gene Expression Assays (Thermo Fisher) and sequences of primer and probe genes are listed in Table 1.

Statistical Analysis: Results have been expressed as mean ± standard deviation. The analysis of the differences between groups was performed using one-way ANOVA with Šidák correction (for a single timepoint) or two-way ANOVA with Tukey's multiple comparison test (for two or more timepoints). Presented results were obtained from experiments that were repeated at least three times, using different donors each time. All statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc.). Asterisks denote statistical significance as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns = no significance.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

C. Plank is a co-founder of Ethisis.

Data Available Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

BMP-7, bone regeneration, cmRNA, granular hydrogels, hMSCs, microgels, osteogenesis

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