Attachment of Ultralow Amount of Engineered Plant Viral Nanoparticles to Mesenchymal Stem Cells Enhances Osteogenesis and Mineralization

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Hydrogel-based materials are widely used to mimic the extracellular matrix in bone tissue engineering, although they often lack biofunctional cues. In the authors’ previous work, Potato virus X (PVX), a flexible rod-shaped biocompatible plant virus nanoparticle (VNP) with 1270 coat protein subunits, is genetically modified to present functional peptides for generating a bone substitute. Here, PVX is engineered to present mineralization- and osteogenesis-associated peptides and laden in hydrogels at a concentration lower by two orders of magnitude. Its competence in mineralization is demonstrated both on 2D surfaces and in hydrogels and the superiority of enriched peptides on VNPs is verified and compared with free peptides and VNPs presenting fewer functional peptides. Alkaline phosphatase activity and Alizarin red staining of human mesenchymal stem cells increase 1.2–1.7 times when stimulated by VNPs. Engineered PVX adheres to cells, exhibiting a stimulation of biomimetic peptides in close proximity to the cells. The retention of VNPs in hydrogels is monitored and more than 80% of VNPs remain inside after several washing steps. The mechanical properties of VNP-laden hydrogels are investigated, including viscosity, gelling temperature, and compressive tangent modulus. This study demonstrates that recombinant PVX nanoparticles are excellent candidates for hydrogel nanocomposites in bone tissue engineering.

1. Introduction

Bone is an extraordinary tissue as well as an organ with a hierarchical structure composed of organic, inorganic, and aqueous phases. Bone tissue engineering has become one of the most common approaches for bone healing by exploiting scaffold, cell, and bioactive factors to mimic native chemical and physical cues, as well as to mimic the hierarchy of bone tissue ranging from molecular composition to extracellular micro/nanostructure. Hydrogels are biomaterials frequently used in this field, acting as a cell carrier, an extracellular matrix (ECM) scaffold for cell-mediated tissue-regeneration and as a cargo of bioactive molecules for stimulation of cells and environment. Given their critical contribution to bone homeostasis and disease, many applications have employed mesenchymal stem cells (MSC) to regenerate bone tissue. MSCs are strongly regulated by their surrounding microenvironment, which makes scaffold design and additive bioactive factors in their development challenging. Non-collagenous proteins (NCP) play a vital role in the processes of mineralization and osteogenic differentiation of MSCs in bone tissues. To mimic physiological mineralization, many studies have combined NCPs with bone substitutes such as bone sialoprotein (BSP), osteopontin, and osteocalcin. Some studies have selected certain functional protein fragments and applied them to the bone replacement by incorporation of...
polymers\textsuperscript{[6]} or delivery via nanoparticles/vesicles.\textsuperscript{[7]} However, such systems exhibit some drawbacks, including a low concentration of proteins/peptides and low retention time of nanoparticles within the polymer, which lead to a loss of the desired function.

To cope with the difficulties of retention and cell signaling, we propose a robust and stable composite—Potato virus X (PVX)—in combination with hydrogel-based materials for bone substitution. PVX is a flexuous, rod-shaped plant virus particle approximately 515 × 15 nm in length and composed of 1270 coat protein subunits (CP) helically arranged around a plus-sense RNA.\textsuperscript{[8]} Viral nanoparticles (VNPs) can be genetically modified to precisely present desired peptides or other molecules either by genetic engineering, chemical conjugation, or a combination of the two adapted to specific demands.\textsuperscript{[9]} In contrast to many artificial nanomaterials, VNPs offer a precise nanoscaled structure with dimensional and organizational uniformity.\textsuperscript{[10]} Moreover, filamentous particles have the advantage of greater surface area compared to spherical particles such as Cowpea mosaic virus, thus providing more potential sites for targeted molecule exposition.\textsuperscript{[11]} This feature enables the feasibility of presenting highly concentrated functional peptides in a local area, which can significantly enhance their effectiveness.\textsuperscript{[12]} Plant virus nanoparticles with multivalent protein surfaces have facilitated many medical applications, including vaccination, biosensing, and tissue engineering, by functionalizing them with compounds.\textsuperscript{[13–15]} In particular, VNPs have also been used as building blocks to tune cellular responses such as cell adhesion and differentiation.\textsuperscript{[16]} In our previous work, we engineered PVX to display cell-adhesive arginine-glycine-aspartate (RGD) peptides, which enhanced focal adhesions of human MSCs (hMSC) on a VNP-coated surface and improved cell attachment to a non-adhesive agarose hydrogel, resulting in an ECM-like microenvironment.\textsuperscript{[17]} We also genetically modified PVX to present a mineralization-inducing peptide (MIP), which induced hydroxyapatite (HAP) nucleation and resulted in bundle-like structures. In this work, we utilized newly constructed VNPs at a very low concentration to mimic NCP function for hMSC osteoinduction and biomineralization in hydrogels. The concentration of VNPs used in this work is lower than in our previous work and in other VNP applications\textsuperscript{[11,16]} by two orders of magnitude. This reduces not only the required quantity for VNP production but also the probability of causing immune response in hydrogel implants, even though that probability is already very low. Polyglutamic acid (E8) and hydroxyapatite-binding peptide (HABP) are two peptides applied in this study that showed a more profound effect than the MIP3 peptide in our previous study. E8 originates from BSP, which is expressed in mineralizing connective tissues and is also a potent nucleator of hydroxyapatite.\textsuperscript{[5]} HABP was found by Gungormus et al. by means of a successful screening of a peptide-phage library.\textsuperscript{[19]} PVX-E8 and PVX-HABP were genetically constructed, produced by molecular farming via infection of Nicotiana benthamiana plants as hosts, then isolated. We combined these viral particles with hMSCs in hydrogels. The interaction between the viral particles and hMSCs was of interest, including in terms of cell viability, cell attachment, distribution, and osteogenic differentiation. We examined these features first on 2D surfaces, including coating a plate with virus and immersing it in cell culture medium. Afterward, we incorporated the viral particles into hydrogels as a 3D environment, specifically agarose hydrogels, due to the benefits of their low cost, ease of handling, and bioinertness. The hybrid of viral particle and hydrogel was characterized by VNP release rate, hydrogel viscosity, gelation temperature, and compressive tangent modulus. The mineralization effect of VNPs was verified by simulated body fluid (SBF) immersion. In addition, VNPs with less coverage of peptides were constructed in order to demonstrate an improved mineralization effect brought about by a high local concentration of peptides.

2. Results

2.1. Plant Virus Production and Analysis

In order to mimic the function of NCPs, recombinant PVX nanoparticles presenting different MIPs were designed (Table S2, Supporting Information). Two peptides well known to contribute to the mineralization process were chosen: 1) A polyglutamate sequence (E8)\textsuperscript{[5]} was modified with additional repetitive positive amino acids arginine and lysine (Lys) in order to adjust the isoelectric point (IEP) of the target peptide to be exposed. These amino acids are well-suited as they also control the nucleation and shape of crystals.\textsuperscript{[20]} The IEP adjustment was essential for the nanoparticles to be assembled and for systemic infection of the N. benthamiana plants;\textsuperscript{[21]} 2) A HABP according to Gungormus et al.\textsuperscript{[19]} The encoding sequences were genetically fused to the 5′ end of the PVX cp because the CP N-terminus projects to the outer particle surface.\textsuperscript{[22]} This strategy produces PVX nanoparticles that present the E8 or HABP on each of the 1270 CP subunits.

To investigate the concentration-dependent effect of the target peptides on mineralization and cell responses, PVX nanoparticles with different amounts of peptide coverage were produced (Table S2, Supporting Information). Therefore, two distinct Foot-and-mouth disease virus (FMDV) 2A sequences were introduced that enable a ribosomal skip during particle production resulting in either 10% or 30% fusion proteins.\textsuperscript{[23,24]}

The PVX vectors were used for particle production via infection of N. benthamiana, resulting in successful systemic infection of plants. The plants showed a dwarf phenotype and curling of leaves typical for PVX infection (Figure S1, Supporting Information). The expression of fusion proteins was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot probed with anti-PVX antibody (data not shown).

Recombinant PVX-MIPs were purified using a modified protocol from the International Potato Centre. The expression of recombinant PVX-MIPs produced yields of \( \sim 30–390 \text{ mg kg}^{-1} \) infected leaf material (Table S2, Supporting Information). All fusion proteins were detected at anticipated molecular weights (PVX-HABP: 25.6 kDa, PVX-E8: 26.8 kDa, PVX-HABP-2A 30%: 27.4 kDa, PVX-HABP-2A 10%: 27.9 kDa, PVX-E8-2A 10%: 29.2 kDa) in the Coomassie stained gel and the corresponding specific western blot (Figure 1A). PVX-HABP and PVX-E8 showed some additional bands representing degradation products. For VNPs produced by the FMDV 2A strategy, two bands were detected as expected: one for the fusion protein and one for the PVX CP with 25.1 kDa, both of which contribute to particle assembly. Compared to PVX-HABP, PVX-E8, and PVX-HABP-2A 30%, only a small amount was detected for fusion proteins of PVX-HABP-2A 10% and PVX-E8-2A 10%.
To verify recombinant MIP sequences at the genomic level, viral RNA of purified VNPs was analyzed by reverse transcription polymerase chain reaction (RT-PCR) and sequencing (Figure 1B). As expected, wild-type (WT) PVX particles showed a 502-bp product with correct sequence information and there were no bands visible in other control lanes representing non-infected *N. benthamiana* leaf extract and water. Sequence analysis for PVX-HABP (≈548 bp), PVX-E8 (≈572 bp), PVX-HABP-2A 30% (602 bp), and PVX-HABP-2A 10% (614 bp) confirmed functional CP fusions. In contrast, a deletion from the 5’ end was detected for PVX-E8-2A 10% (638 bp), resulting in partial loss of the E8 and FMDV 2A sequence. Therefore, this construct was excluded from further experiments.

Particle morphology and mineralization was verified by transmission electron microscopy (TEM) (Figure 2). To accomplish this, particles and controls labeled with immunogold conjugates were incubated in SBF according to Kokubo’s bioactivity assay for 7 days to investigate their mineralization potential.[25] The controls, representing non-infected *N. benthamiana* leaf extract and WT PVX particles, showed no nucleation centers or crystals in SBF, as expected. Synthetic HABP and E8 peptides produced small nucleation centers and ≈500 × 500 nm platelet-like crystals, respectively. Recombinant PVX particles displaying negatively charged E8 residues (PVX-E8) were able to mineralize plate-like crystals with a shape of ≈500 × 1000 nm in SBF. VNPs with HABP presented on every single CP subunit nucleated calcium crystals with a regular shape of ≈30–100 nm along their long axis. Interestingly, PVX-HABP-2A 30% and 10% showed plate-like crystals of 250 × 500 nm. These experiments confirmed the ability of recombinant MIP displaying PVX nanoparticles to promote crystal formation in vitro.

2.2. VNP and Cell Interaction

Cell attachment on various VNP-coated surfaces was detected (Figure 3A). Compared to the no-coating tissue culture plastic surface, the WT PVX-, PVX-E8-, and PVX-HABP-coated surfaces were less ideal for hMSCs to attach to. The cell attachment on VNP-coated surfaces is concentration dependent, which can be seen by the absorbance of cells on PVX-E8-coated surfaces at various densities (Figure 3B). At 0.048 pmol cm⁻² PVX-E8 coating
Figure 3. VNP and cell interaction in 2D cell culture. Cell attachment evaluation by crystal violet assay. A) Absorbance from cells on different VNP-coated surfaces. B) Absorbance from cells on PVX-E8-coated surfaces at different coating concentrations. C) Normalized Alizarin red staining values of hMSCs induced toward osteogenic differentiation on PVX-E8 coated surfaces. D) ALP assay of hMSCs osteogenic differentiation on VNP-coated surfaces on days 7, 14, and 21. (A–D) Data are presented as means ± standard deviations (n = 3). Data analyses were performed using Student’s t-test and statistical significance was marked as *p < 0.05, **p < 0.01, and ***p < 0.005. E) Fluorescent microscopy images of hMSC and VNP distribution on 2D surface. hMSCs were labeled with Vybrant DiI (orange), VNPs were labeled with anti-PVX and secondary antibody (AlexaFluor488, green), and cell nuclei were stained with DAPI (blue).

density, the cell attachment effect is similar to the no-coating control, and the values of both concentrations are significantly higher than those with 0.48, 1.5, and 2.4 pmol cm$^{-2}$ coating densities. PVX-E8 coating concentration at 0.48 pmol cm$^{-2}$ is also higher than the two lower concentrations.

Calcium deposition of hMSCs was detected on days 14 and 21. The values of the staining in the osteogenic induction medium groups were normalized to the values in the growth medium groups (Figure 3C). PVX-E8 showed higher calcium deposition than the negative control, with a significant difference on day 14.

The alkaline phosphatase (ALP) activity of PVX-E8, an early stage marker of osteoinduction, was significantly higher than the activity of the control group on day 7 (Figure 3D). Both Alizarin red staining and ALP assay led to a similar conclusion that PVX-E8 was effective in inducing osteogenic differentiation of hMSCs.

In the experiment with hMSC and VNP distribution on a 2D surface, WT PVX and PVX-HABP were evenly distributed on the entire cell culture areas, while PVX-E8 tended to attach to cell surfaces (Figure 3E). A large proportion of PVX-E8 was
Figure 4. VNP-laden hydrogel characterization. A) VNP release rate from hydrogels. Data are presented as means ± standard deviations (n = 4). Data analysis was performed using Student’s t-test and statistical significance was marked as *p < 0.05, **p < 0.01, and ***p < 0.005. B) Viscosity and gelling temperatures of VNP-laden agarose hydrogel. C) Compressive tangent modulus of VNP-laden hydrogels. (B, C) Data are presented as means ± standard deviations (n = 3). Data analyses were performed using one-way analysis of variance followed by Bonferroni’s post hoc test. Statistical significance was marked as *p < 0.05, **p < 0.01, and ***p < 0.005. D) EDX analyses of VNP-laden hydrogels treated in mineralization condition. E) EDX analyses of various concentrations of PVX-HABP-laden hydrogels treated in mineralization condition. F) EDX analyses of PVX-HABP, PVX-HABP-2A 30%, PVX-HABP-2A 10%, and synthetic HABP-laden hydrogels treated in mineralization condition. In EDX diagram, x-axis represents energy (keV), while y-axis represents counts.

detected on the cell surfaces, including cell bodies and filopodia-like extensions, while a small amount of PVX-E8 was detected on the cell culture plate.

2.3. Characterization of VNP-Laden Hydrogel

To test whether the particles are stably incorporated into the hydrogels, the VNP release rate was examined (Figure 4A). WT PVX could not be detected during the 5 days, whereas PVX-HABP released around 6% and 10% of its total amount on days 2 and 3, respectively. Although enzyme-linked immunosorbent assay (ELISA) allowed the detection of rather small amounts of particles (2.5–0.25 ng µL⁻¹), most samples were below the detection range (The individual values are listed in Table S5, Supporting Information). PVX-E8 was not included in the graph due to the limited capability of ELISA detection, which will be elaborated in the discussion.
Figure 5. VNP and cell interaction in hydrogels. A) hMSC viability in VNP-laden hydrogels. Data are presented as means ± standard deviations from three independent donors (each donor contained sample size \( n = 3 \)). Data analysis was performed using Student’s t-test and statistical significance was marked as \( *p < 0.05 \), \( **p < 0.01 \), and \( ***p < 0.005 \). Two-photon laser scanning microscope (TPLSM) images of hMSCs embedded in VNP-laden hydrogels. Concentrations of encapsulated VNPs were B) \( 6 \times 10^{-8} \) M, C) \( 6 \times 10^{-10} \) M, and D) \( 6 \times 10^{-12} \) M. VNPs were labeled with anti-PVX antibody and secondary antibody (AlexaFluor488, green); lipophilic membrane structure of cells was stained with Vybrant DiD dye (red); cell nuclei were stained with DAPI (blue). (B) VNPs and cells were evenly distributed in agarose hydrogels. After 7 days, VNPs remained in hydrogels. (C, D) VNPs encapsulated at \( 6 \times 10^{-10} \) M showed more attachment to cells than VNPs encapsulated at \( 6 \times 10^{-12} \) M. E) Confocal laser scanning microscopy images of a single hMSC in a VNP-laden hydrogel. VNPs were encapsulated in hydrogels at \( 6 \times 10^{-10} \) M. The labels are the same as previous description in the two-photon microscopy images. F) ALP assay of hMSCs induced toward osteogenic differentiation in VNP-laden hydrogels for up to 21 days. VNPs were encapsulated in hydrogels at \( 6 \times 10^{-10} \) M. Data are presented as means ± standard deviations (\( n = 4 \)). Data analysis was performed using Student’s t-test and statistical significance was marked as \( *p < 0.05 \), \( **p < 0.01 \), and \( ***p < 0.005 \).

Compared to the negative control, the viscosities of WT-, PVX-E8-, and PVX-HABP-laden hydrogels showed no significant change. The viscosities were around 8–12 mPa s in all groups (Figure 4B), indicating that the addition of VNPs at these specific concentrations did not change the viscosity of the hydrogels. The gelling temperatures of various VNP-laden agarose stayed at around 28 °C in all groups and did not show significant differences to each other. Also, there was no considerable change in the compressive tangent moduli of agarose hydrogels laden with different types of VNP (Figure 4C).

The mineralization effect of VNP-laden hydrogels was monitored (Figure 4D–F). Among all of these, only PVX-HABP-laden hydrogels showed the signals of phosphate and calcium, indicating production of calcium phosphate in the hydrogels. In addition, all the PVX-HABP-laden hydrogels with various concentrations displayed the signals of phosphate and calcium in the EDX diagrams (Figure 4E). The calcium phosphate production in the hydrogel with the highest PVX-HABP concentration (\( 6 \times 10^{-8} \) M) was visualized in the scanning electron microscopy (SEM) image (Figure S4B, Supporting Information). PVX-HABP with 30% and 10% coverage as well as synthetic HABP were tested under the same conditions (Figure 4F), however none of these displayed calcium signals in the energy-dispersive X-ray spectroscopy (EDX) diagrams.

2.4. Interaction of VNP-Laden Hydrogels and Cells

hMSC was initially assessed for its viability in VNP-laden hydrogels (Figure 5A). The viabilities in WT PVX-, PVX-E8-, and PVX-HABP-laden hydrogels were similar to the no-virus control, which was above 90% indicating good cytocompatibility of WT PVX and the modified VNPs.

VNPs and cells were evenly distributed in the hydrogels in various groups (Figure 5B–D). After 7 days, VNP mostly remained in the hydrogels of all groups (Figure 5B), which corresponded to the VNP release rate result (Figure 4A) and demonstrated a high retention time of VNP in hydrogels. The images also revealed that VNP were located in no-cell spaces as well as tightly associated with hMSCs. In order to have a clear view of the interaction of VNPs and hMSCs, confocal laser scanning microscopy was employed (Figure 5E). The hMSCs were surrounded by modified VNPs as well as WT PVX (the 3D constructs are included in Supporting Information as a video). Furthermore, VNPs were also encapsulated in lower concentrations of \( 6 \times 10^{-10} \) M (Figure 5C) and \( 6 \times 10^{-12} \) M (Figure 5D). Both groups showed VNP attachment to the cells, with the VNPs encapsulated at \( 6 \times 10^{-10} \) M showing more attachment to cells than those encapsulated at \( 6 \times 10^{-12} \) M.
The hMSCs embedded in VNP-laden hydrogels were under osteogenic differentiation induction for up to 21 days and the ALP activity was measured on days 7, 14, and 21 (Figure 5F). PVX-E8 and PVX-HABP were significantly higher than the no-virus control on day 14 and PVX-HABP was statistically higher than the control on day 21, suggesting an enhanced osteoinduction with VNP-laden groups.

3. Discussion

3.1. VNP Production and Mineralization In Vitro

To support cell interaction between the surface of a biomimetic nanocomposite and human tissue, appropriate physical and cellular signals are needed. In nature, cell adhesion and proliferation as well as HAP mineralization are promoted and controlled by certain polar and charged amino acids of the NCPs located in the ECM.[26] Based on the successful use of plant viruses in medical approaches,[27] we created mineralization-inducing PVX nanoparticles presenting two different peptides known to contribute to the mineralization process: HABP, which binds strongly to HAP,[19] and the BSP-derived sequence for E8, which is responsible for HAP nucleation.[28,29] Adding sequences increases the genetic load of PVX, which can interfere with particle assembly and local and long-distance movement within the plant.[30,31] Major considerations in this context are the sequence length and the restricted IEP range of CP fusions, which must fall within certain ranges. The size restriction is usually 8.5 kDa for direct PVX CP fusions,[32] although a 13.5 kDa fusion was also expressed recently,[33] and the IEP must be between 5.2 and 9.2.[21] For these reasons, only small protein sequences of NCPs were chosen and the E8 sequence was adjusted with repeats of basic amino acids lysine and aspartate. To investigate a concentration-dependent effect on the mineralization process and cell interaction, we also generated particles partly decorated with the desired MIP by using two different FMDV 2A sequences, resulting in a 10% or 30% coverage. These sequences are often incorporated when the target peptide composition or length is disadvantageous for particle assembly and systemic movement, as the FMDV 2A sequence achieves a ribosomal skip during translation thus providing additional non-fused CPs that facilitate intact particle assembly.[34] This strategy is widely used to avoid size limitation with PVX and tobacco mosaic virus (TMV) vectors.[12,34,35]

Engineered VNPs were specifically analyzed at the protein and genetic level (Figure 1) because some additional degradation products were detected for PVX-HABP and PVX-E8 and only a very small amount for PVX-E8-2A 10%. All particles showed correct sequence information of encapsidated RNAs, thus minor degradation products reflect proteolytic degradation. However, PVX-E8-2A 10% indicates a deletion from the 5’ end representing a loss of the E8 and part of the 2A sequence, which might explain the fewer functional particles detected in western blot and cDNA analysis. Previous studies with recombinant viral vectors have shown that large inserted sequences or highly repetitive sequences, such as duplicated subgenomic-like promoters, are instable and rapidly eliminated because these sequences are non-essential for virus multiplication, assembly, or long-distance movement.[38,39] With the addition of E8 and the 2A sequence this limitation might be reached, leading to backmutation or inefficient virion assembly and loss of the desired function. Therefore, PVX-E8-2A 10% was excluded from further experiments.

Engineered VNPs were incubated in SBF, a favored biocompatible material that closely mimics the mineral phase of human bone,[40] in order to investigate their mineralization potential in vitro. Depending on the presented amino acids, different crystal morphologies were expected.[20] Based on their charge, these amino acids attract Ca\(^{2+}\) and PO\(_4\)\(^{3-}\) ions, nucleating and developing into HAP crystals.[26,41] Most research has focused on the effect of negatively charged aspartic (Asp, D) and glutamic acid (Glu, E) representing putative Ca\(^{2+}\) interaction sites, and only a few studies have focused on the effect of glycine (Gly, G), which is present in HABP. Nevertheless, Tao et al. demonstrated the formation of rod-like crystals in the presence of Gly.[42] The synthetic HABP used in our in vitro biomimeralization assay showed a few nucleation centers under electron microscopy, while for PVX-HABP particles crystals of a regular angular shape were observed. When VNPs presented a less dense HABP concentration (PVX-HABP-2A 30% and 10%), plate-like crystals similar to bone apate were formed, possibly due to the predominant presence of WT CP comprising HAP-modulating serine (Ser) residues. Similar results were shown earlier in studies using direct CP fusions with MIP3 containing Glu, Asp, and Ser residues,[17] which function as major nucleation sites and therefore display strong affinity sites for HAP. Furthermore, a high local concentration of peptides on small scaffolds like silk backbones or biocompatible VNPs favor a greater control over mineralization.[17,43]

The spatial organization and constitution of the created artificial ECM will greatly influence the cell responses and mineralization potential.[44] Bone ECM consists predominantly of collagen type I, and only &5% are NCPs such as osteocalcin, osteopontin, osteonectin, and fibronectin.[45] The latter contains the cell-adhesion motifs RGD and PHSRN required for a stable binding of the α5β1 cell receptor, separated by ≈4 nm in its native conformation.[46,47] This is approximately the distance of the helical pitch of PVX CP subunits (3.45 nm), highlighting PVX as an excellent scaffold for NCP sequences.

Another promising MIP candidate was found in peptides containing poly-Glu sequences since Glu and Asp not only attract HAP but also assist in regulation of crystallinity growth by an inhibitory effect.[44] Here, plate-like structures were observed for synthetic E8 and PVX-E8, as also reported by Tavaghi and Cerruti[20] and Polini et al. for poly-Glu sequences.[49] The restricted IEP range reported for successful PVX infection would potentially exclude eight glutamic acid residues (IEP 2.8, fusion with CP: 4.6). Thus, PVX-E8 particles needed to be tailored with charge-compensating Asp and Lys, selected for their supporting effect on HAP binding and nucleation.[20]

3.2. VNP and Cell Interaction in Both 2D and 3D Cultures

Plant viruses prevail in many places and we therefore encounter them on a daily basis.[15] They are non-toxic, biocompatible, biodegradable, and non-infectious to mammals. The toxicity and immune response of PVX-based materials have been examined in many studies,[9,15] In this work, the cell viability was also monitored. hMSC viability was tested on VNP-coated surfaces (Figure S3, Supporting Information) and in VNP-laden hydrogels
(Figure 3A) and showed a very high viability above 90% after being cultured with VNPs for up to 7 days. This result laid a good foundation for further cell-related experiments. In addition, studies have shown that PVX maintains an excellent stability in biological media. Although parts of the N-terminal CP segment may be degraded by trypsin treatment, PVX maintains its filamentous structure by hydrophobic and electrostatic interactions.[50,51] Thus the PVX RNA is protected from degradation under physiological conditions by encapsidation.[52,53] Compared to synthetic nanoparticles, plant viruses can be stored in infected leaf material at −80 °C or as purified solutions for months, facilitating a long-term utilization. Therefore, plant viruses have been widely applied in tissue engineering and many other biomedical applications.[15,16,54]

Research studies have demonstrated that MSCs can respond to micro/nanostructures that resemble the architecture found in natural bone, resulting in more effective proliferation and differentiation along an osteogenic lineage.[2] The cell attachment assay revealed an inferiority of VNP-coated surfaces to the no-VNP control and the cell attachment decreased along with the increasing PVX-E8 coating density (Figure 3A,B). This outcome is to be expected due to the fact that no adhesion-promoting cue was conjugated on the VNPs. Metavarayuth et al. also showed that BMSCs only weakly attached to the viruses TMV and PVX, but improved osteogenesis after induction.[35] It is noteworthy that after a longer culturing period of 24 h the adhered hMSCs on the PVX-E8 surface increased, which is also the reason why only the PVX-E8 group was selected for further 2D experiments. To enhance cell attachment with PVX, our previous study showed an improvement using PVX engineering with RGD peptides.[17]

Studies have demonstrated osteogenic differentiation of MSCs on virus-coated substrates, which generate nanotopographical signals and/or chemical cues that alter the cell behavior, including phases modified with RGD,[56] TMV,[57,58] TMV modified with phosphates,[61] and PVX modified with RGD, and MIPs.[17] In this work, the osteogenic differentiation of hMSCs was evaluated on PVX-E8-coated surfaces by Alizarin red staining and ALP assay, both of which showed a significant increase compared to the control (Figure 3C, D). PVX-E8 was also encapsulated in cell-embedded VNP-laden hydrogels (Figure 5F) and an enhanced ALP activity was shown. The effect of promoting osteogenic differentiation by E8 peptides was clearly displayed. PVX-HABP-laden hydrogel also presented an increased ALP activity, showing that the hydroxyapatite binding capacity of the VNPs promoted the osteogenic differentiation of hMSCs. Compared to the PVX engineered with MIP3 from our last work,[17] we have demonstrated a considerably improved osteogenesis with a lower amount of PVX-E8 and PVX-HABP.

In the immunostaining images, PVX-E8 showed a tendency toward cells on a 2D surface (Figure 3E), while WT, PVX-E8, and PVX-HABP closely adhered to cells in hydrogels (Figure 5B–E). This characteristic of PVX offers an advantage in stimulating cells by the engineered functional peptides at a very close proximity. It is, however, unclear why VNPs have an affinity with cells and further investigation should be carried out in the future. Notably, VNPs presented only on surfaces without entering the cells, showing no sign of endocytosis. Nevertheless, this could be changed by engineering different peptides; for example, PVX-presenting RGD peptides could be found inside cells (Figure S5, Supporting Information). This observation is in accordance with the results of Tian et al., who utilized cRGD as an integrin-binding peptide to increase the cellular uptake of TMV for doxorubicin delivery.[62]

The TPLSM images revealed the cell-VNP distribution in hydrogels with various VNP concentrations: 6 × 10⁻⁸, 6 × 10⁻¹⁰, and 6 × 10⁻¹² m (Figure 5B–D). Compared to the hydrogel with the highest VNP concentration, the hydrogel with 6 × 10⁻¹⁰ m VNPs contained fewer VNP clusters with a more suitable density, but the amount of closely attached VNPs on cells still remained rather high. This result showed that 6 × 10⁻¹⁰ m was the most suitable concentration for close stimulation.

3.3. Characterization of VNP-Laden Hydrogels

A biomolecule delivery system for tissue engineering requires a controlled local release to maintain the biological effects over a long period of time, avoid a burst release and limit the diffusion of biomolecules to non-target sites.[63] In our system, WT PVX was not detected in the immersed solution since it was below the detection limit, whereas PVX-HABP released around 18% of the total amount indicating that more than 80% remained inside the hydrogels (Figure 4A). The polyclonal anti-PVX antibody used in ELISA detects several epitopes on the surface of PVX CP, which is also the position where the fusion peptides E8 or HABP are located. It is probable that the fusion peptides decrease the efficiency of the antibody by hindering it from binding to the target. This is shown in the standard solutions (Table S3, Supporting Information), in which the detection of PVX-HABP was lower than WT PVX and PVX-E8 was barely detected under the chosen conditions. Additionally, the immunostaining images showed that the majority of VNPs remained in hydrogels after a 7-day culturing period (Figure 5B). Luckanagul et al. produced TMV-laden alginate hydrogels by mixing fluorescently labeled TMV into the solution before freeze-drying.[54] The release profile showed a 13% release after 24 h in solution. In comparison with their result, the release rate from our system was either slightly higher (18% for PVX-HABP) or much lower (almost 0% for WT PVX), which demonstrates a long retention time of VNPs maintaining biofunctional effects in hydrogels.

Mechanical properties are one of the important factors that influence cell behavior and tissue compatibility and are tunable by altering physical or chemical composition.[2] Zheng et al. demonstrated an increase in storage and compressive modulus of a poly(poly(ethylene glycol) methyl ether acrylate) hydrogel by increasing the amount of tobacco mild green mosaic virus, [11] and Southan et al. showed an increase in the stiffness of poly(ethylene glycol) diacrylate hydrogels by incorporating covalent force between TMV and the polymer.[64] Nevertheless, TMV reduced the mechanical strength of hyaluronic hydrogels,[65] showing that VNPs can influence different hydrogel types in different ways. In this work, no significant change in the stiffness of the VNP-laden hydrogels was observed (Figure 4C). Different plant virus topologies and concentrations might also lead to different effects on hydrogels. TMV is a rigid virus, whereas PVX is a flexible one that might not be sufficient for a mechanical change. Also, the amount of laden VNPs in this work is approximately 0.002% w/v, which is much lower than the 0.1–5% level of Zheng et al. and
the 0.3% in the Southan et al. study. (To equalize the number of VNP s, we used the concentration 6 × 10⁻¹⁰ m.) Besides, there is no covalent bond between VNP s and hydrogel in our system. Furthermore, the viscosity and the gelling temperature of the VNP-laden hydrogels did not change significantly (Figure 4B). The work of Sen et al. demonstrated an increased viscosity of agarose hydrogel by the addition of calcium phosphate, resulting in a higher gelation temperature.⁶⁶ Compared to inorganic particles, PVX is a protein-based material that might not be adequate to cause a difference in local elastic moduli in a composite matrix.

Calcium phosphate has been utilized to mimic the inorganic components of bone and incorporated into biomaterials to promote osteoinductivity. SBF has been applied in many biomaterials to obtain biomimetic mineral properties and to assess bioactivity.⁶⁷ Among all the VNP-laden hydrogels (Figure 4D), PVX-HABP was the only one that induced mineralization inside the hydrogels, and the minerals were on a micrometer scale at their highest concentration (6 × 10⁻⁸ m) (Figure S4B, Supporting Information). Unlike the mineralization on a 2D surface (Figure 2), PVX-E8, PVX-HABP-2A 30%, PVX-HABP-2A 10%, and synthetic HABP did not present a calcium signal inside hydrogels (Figure 4F). This is probably because the amount of nucleated HAP was not great enough to be detected. The nucleated HAPs in hydrogels might be lost due to the diffusion of liquid and washing steps for the sample preparation. This was not the effect for PVX with a 100% coverage, indicating that the amount of displayed HABPs might have an effect on HAP binding and thus presentation in hydrogels. Synthetic HABP-laden hydrogel might be subject to a loss of desired function due to a low retention time of free peptides. This result illustrated the benefits of PVX usage in tissue engineering, where it acts as a carrier for the delivery of peptides and provides a high concentration of peptide in a local area.

In comparison with the MIP3 from our previous study,¹⁷ the newly engineered recombinant PVXs in this work showed superiority in mineralization and osteoinductive differentiation, and reached a higher potential for plant virus incorporation in bone tissue engineering with an even lower amount of VNPs. PVX engineered with RGD peptide and PVX-RGD-MIP3 from the last work showed improved cell attachment and increased focal adhesion. Hence, a prior idea was to engineer PVX with both RGD and E8/HABP peptides on a single particle to achieve a synergistic effect. However, the plant infection was not successful, possibly due to steric effects preventing particle assembly. An intervening FMDV 2A sequence might solve this problem, although this strategy would provide a reduced amount of target peptides compared to a direct genetic fusion. An alternative approach would be loading PVX-RGD and PVX-E8/PVX-HABP together in a hydrogel to realize the full potential for cell attachment, osteogenic differentiation and mineralization in a hydrogel construct.

5. Experimental Section

5.1. Design and Engineering of Plant VNPs

DNA was resolved by 1.2% w/v agarose gel electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris base, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA)). PCR products were isolated from agarose gels using Wizard Plus SV Miniprep DNA Purification System (Promega, USA). The PureYield Plasmid Miniprep System or PureYield Plasmid Midiprep System (Promega) were used to isolate DNA. MSB Spin PCRapace (Stratec, Germany) was used for buffer exchange in successive restriction digests.

PVX-based expression constructs were produced by introducing the codon-optimized target sequences to the 5’-end of the PVX cp gene. To accomplish this, a PCR with specific primers (sequences are listed in Table S1, Supporting Information) was carried out using Pfu Polymerase (Promega) prior to 3’ end adenyla-

ligation, which was achieved by incubation for 15 min at 72 °C (25 µL isolated PCR product, 8 µL 5× colorless Go Taq reaction buffer, 1 µL dATPs 10 nmol, 0.4 µL GoTaq G2 polymerase, 5.6 µL H₂O dest.). The adenylated products were transferred to the topoiso-

merase I-activated pCR 2.1-TOPO vector (Thermo Fisher Scientific, USA) and transformed into competent TOP10 cells according to the manufacturer’s recommendations. A control PCR was carried out using primers M13-fw and M13-rev. The resulting vectors pCR2.1 Nhel-E8-CP-Spel, pCR2.1 Nhel-HABP-CP-Spel, pCR2.1 Nhel-E8-Xmal, and pCR2.1 Nhel-HABP-CP-Xmal were digested using restriction enzymes (New England Biolabs, USA) given in the construct names according to the manufacturer’s recommendations. Fragments E8-CP or HABP-CP were ligated into pTCSIIe,⁶⁸ which was digested with Nhel and SpeI. To produce VNPs with target peptide coverages of 10% and 30%, respectively, fragments of E8 or HABP were transferred to pPVX-

iLOV-2A-CP-10% or -30% (the authors’ unpublished data) restricted with Nhel and BspEI. Vectors pTCSIIe, pPVX-iLOV-2A-

CP-10% or -30% were dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs, USA) to avoid self-

ligation. Ligation was carried out at 16 °C for 16–18 h using T4 DNA ligase (Promega, USA). Ligation products were precipitated with ethanol and glycogen before electroporation of Escherichia coli DH5α cells. The pPVX-derived vectors were selected on lysogeny broth (LB) agar plates supplemented with 100 µg mL⁻¹ ampicillin. Transformation was confirmed by control PCR with primers CX1 and CX2, or 2A-10% or 2A-30%. The components for the design of recombinant PVX vectors are listed in

4. Conclusion

We successfully produced two different PVX nanoparticles to present more than a thousand osteogenesis- and mineralization-associated peptides by gene modification. PVX-E8 promoted osteogenic differentiation of hMSCs on surfaces and a tendency to adhere on spread hMSCs. After incorporation into hydrogels, the mineralization effect of PVX-HABP was proven by the detection of calcium phosphate signals, which were absent both from PVXs with less HABP (10% and 30% coverage) and synthetic HABP. This finding suggests that a highly concentrated HABP displayed on a PVX particle exerts a profound effect in hydrogels. Furthermore, PVX-E8 and PVX-HABP demonstrated an improved osteogenesis of hMSCs in hydrogels with a close attachment to cells by an ultralow nanoparticle concentration that is lower by two orders of magnitude than other studies. Given the plentiful advantages it offers, engineered PVX with biomimetic peptides has shown its potential as an effective and efficient composite in bone tissue engineering.
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Table 1. The resulting plasmids were verified by sequencing (Eurofins custom DNA sequencing service).

| Plasmid          | Template     | Forward primer  | Reverse primer |
|------------------|--------------|-----------------|----------------|
| pPVX-E8          | pTCXIII[9]   | NheI-E8-CP      | M13-rev        |
| pPVX-HABP        | pTCXIIIc     | NheI-HABP-CP    | M13-rev        |
| pPVX-E8-2A 10%   | pPVX-E8-CP   | CX2             | E8-XmaI        |
| pPVX-HABP-2A 10% | pPVX-HABP-CP | CX2             | HABP-XmaI      |
| PPVX-HABP-2A 30% | pPVX-HABP-CP | CX2             | HABP-XmaI      |

5.2. Infection and Cultivation of N. benthamiana

Three leaves of 4-week-old N. benthamiana plants were each inoculated with 10 µg of pPVX vectors. Celite 545 (Carl Roth, Germany) was used to gently abrade the leaf surface, and after an incubation of 20–30 min the leaves were rinsed with water to remove it and excess DNA. The plants were cultivated in a phytochamber (12-h photoperiod at 5000–10 000 lux, 22/20 °C day/night, 70% humidity) and harvested 14–21 dpi depending on the progress of infection.

5.3. VNP Purification

Purified VNPs were obtained as previously described by Röder et al.[9] Briefly, 100–150 g plant material was homogenized in two volumes of extraction buffer (0.1 M phosphate buffer (pH 8.0), 0.2% v/v 2-mercaptoethanol and 10% v/v ethanol), filtered through gauze and clarified by centrifugation (7800 × g, 20 min, 4 °C). The supernatant was stirred for at least 1 h at 4 °C with 1% v/v Triton X-100 and then clarified (5500 × g, 20 min, 4 °C). The particles were precipitated with 0.2 M NaCl and 4% PEG (6000–8000) overnight at 4 °C, followed by incubation at room temperature (RT) for 1 h. The precipitate was collected (7800 × g, 30 min, 4 °C) and suspended in 8 mL 0.05 M phosphate buffer (pH 8.0) with 1% v/v Triton X-100, and clarified again (7800 × g, 30 min, 4 °C). The supernatant was loaded onto a sucrose gradient of 45–10% w/v sucrose in 0.01 M phosphate buffer (pH 7.2) with 0.01 M EDTA (96 500 × g, 75 min, 4 °C). Fractions were collected and analyzed using SDS-PAGE. Then, fractions containing the desired particles were combined, diluted in the same volume of 0.01 M phosphate buffer (pH 7.2) and precipitated at 102 600 × g for 5 h at 4 °C. Particles were resuspended in 0.2–0.4 mL phosphate buffer (pH 7.2) overnight at 4 °C. Particle concentrations were calculated by absorption measurement at 260 nm as (Equation (1)).

\[
\text{Concentration} = \frac{\text{Absorption 260 nm} \times \text{Dilution factor}}{\text{Extinction coefficient (2.97)}}
\]  

(1)

5.4. Detection of VNPs

Systemically infected leaves were homogenized in two volumes of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10.1 mM NaHPO₄, 1.5 mM KH₂PO₄, pH 7.4) and cell debris was removed by centrifuge (20 000 × g, 10–15 min, 4 °C). The plant extracts (20 µL) and the purified VNPs (1.5 µg) were analyzed by SDS-PAGE and western blotting using a 12% resolving gel and a 4% stacking gel. The proteins were boiled for 5 min in 5x reducing loading buffer,[69] and separated proteins were either stained with Coomassie Brilliant Blue or transferred to a Hybond-C nitrocellulose membrane (GE Healthcare Life Sciences, USA) using a semi-dry blotting system (Bio-Rad, USA). The membrane was blocked for at least 30 min in 4% w/v skimmed milk in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). Modified VNPs were detected by incubation at RT overnight with a primary anti-PVX antibody (DSMZ, Germany) diluted 1:5000 in PBS, followed by incubation for at least 3 h with an ALP-labeled secondary goat anti-rabbit (GAR) antibody diluted 1:5000 in PBS. The signal was visualized using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (NBT/BCIP) (Carl Roth, Germany) and the reaction was stopped by washing with hot water. The P7712 Prestained Protein Standard (New England Biolabs, USA) was used for sizing.

5.5. Viral RNA Analysis

Purified viruses (5 µg) were mixed with 1 µL oligo-dT primer and incubated for 10 min at 80 °C and for 10 min at 4 °C allowing primer annealing. Virus RNA was reverse transcribed using 5 µL 5x M-MLV reaction buffer, 1 mM dNTPs, 2.5 µL DEPC-H₂O, and 1 µL M-MLV Reverse Transcriptase RNase H Minus Point Mutant (Promega) (30 min at 40 °C, 20 min at 45 °C, 20 min at 50 °C, 20 min at 55 °C, 20 min at 70 °C). The integrity of CP fusions was confirmed by PCR using primers CX3 and CX8 (Table S1, Supporting Information). Ethidium bromide was used to visualize the resolved DNA under UV light in a 1.2% w/v agarose gel electrophoresis in 1× TAE buffer. The resulting RT-PCR products were isolated and sequenced (Eurofins custom DNA sequencing service).

5.6. In Vitro Bioactivity Assay

Hydroxyapatite was formed according to Kokubo’s in vitro bioactivity assay.[24] Therefore, 10–20 µg of engineered VNPs or WT PVX were captured with anti-PVX antibody (DSMZ, diluted 1:100 in water), or synthetic peptides (Life Technologies, Germany) were coated onto nickel grids for 30 min. After washing with water, the grids were blocked with 0.5% w/v BSA (British BioCell, UK) for 20 min, and washed again prior to incubation in 25 mL SBF for 7 days at 37 °C. Mineralized particles were visualized by TEM.

5.7. Transmission Electron Microscopy

For immunogold labeling of purified VNPs, 10 µg of the purified virus was adsorbed to Pioloform-coated nickel grids (Plano, Germany) for 20–30 min at RT. After washing with PBST (PBS, 0.05% v/v Tween-20), the grids were blocked with 40 µL 0.5% w/v BSA (British BioCell) for another 30 min and washed with PBST.
The grids were then incubated with the primary anti-PVX antibody (DSMZ, diluted 1:100 in PBS, incubation 16–18 h) and secondary GAR antibody conjugated to 15-nm gold particles (British BioCell, diluted 1:100 in PBS, 2–4 h). After extensively washing with PBST, PBS, and deionized water, the particles were contrasted with 1% w/v uranyl acetate. TEM was carried out using a Zeiss EM10 microscope (Carl Zeiss AG, Germany).

For the analysis of mineralization assays, recombinant particles were adsorbed to the grids and washed with water. The grids were air-dried and analyzed by TEM without counterstaining.

5.8. VNP Coating on a Surface

VNP solution was diluted and added to a cell culture well plate or coverslips to achieve the concentration of 1.5 pmol cm⁻² after the incubation overnight at RT.

5.9. Human Mesenchymal Stem Cells Culture and Osteogenic Differentiation Induction

Bone marrow-derived MSCs were isolated from human bone marrow aspirate, which was consented to and followed the guidelines of the Ethics Committee of the Faculty of Medicine of RWTH Aachen University (EK 300/13). Cells and specimens were seeded in cell culture flasks and incubated for 1 week at 37 °C in a humidified 5% v/v CO₂ incubator. Subsequently, non-adhered cells were washed away by MSC growth medium change (Mesenpan, PAN Biotech, Germany), whereas adhered cells were in culture and expanded to up to four passages in growth medium, to which was added 2% w/v fetal bovine serum and 1% w/v penicillin/streptomycin.

hMSCs were either seeded at a density of 31 000 cells per cm² in growth medium or encapsulated in 1% w/v agarose at a concentration of 10⁶ or 2 × 10⁶ cells mL⁻¹, and the medium was changed on the following day to osteogenic induction medium (Dulbecco’s Modified Eagle Medium - low glucose (Sigma-Aldrich, USA), with 10% w/v fetal calf serum, 0.05 mm l-ascorbic acid 2-phosphate (Sigma-Aldrich), 10 mm sodium β-glycerophosphate, 100 nm dexamethasone, and 1% v/v penicillin/streptomycin). The medium was changed nine times over a period of 21 days. The formation of calcium accumulations was investigated by qualitative and quantitative Alizarin red staining assay (Sigma-Aldrich). ALP activity was measured with alkaline phosphatase colorimetric assay kit (Abcam, UK) according to the manufacturer’s instructions.

5.10. Cell Encapsulation in VNP-Laden Hydrogels

Cell encapsulated VNP-laden hydrogels were prepared by mixing 3% w/v agarose stock solution (Sigma-Aldrich), cell suspension and VNP solution at 37 °C. 80 µL of the mixture was pipetted into a cylindrical mold in a well plate at RT and formed into a gel during the cooling process. The medium was added to each well after the mold was removed. The final concentrations of the components were 1% w/v agarose, 10⁶ or 2 × 10⁶ cells per mL, and 6 × 10⁻⁶, 6 × 10⁻⁹, 6 × 10⁻¹⁰, 6 × 10⁻¹¹, or 6 × 10⁻¹² m VNP, which are marked in each experimental section.

5.11. Cell Viability

Cell viability was measured using live/dead assay (Sigma-Aldrich) consisting of propidium iodide and fluorescein diacetate in Ringer’s solution. The viability was calculated based on the fluorescent images of live and dead cell numbers.

5.12. Crystal Violet Assay

hMSCs were seeded on the VNP-coated surfaces at 60 000 cells per cm² and incubated at 37 °C for 30 min. Afterward, the cells were rinsed with PBS to remove unadhered cells. Adhered cells were fixed with 1% v/v glutaraldehyde/PBS for 15 min and then washed with PBS. Cells were stained with 0.1% v/v crystal violet solution in distilled water (AppliChem, Germany) for 25 min at RT and washed three to five times with water. The cell-bounded dye was released by adding 50 µL 0.2% v/v Triton X-100 and the absorbance was measured with a spectrometer (SpectraMax M2, Molecular Devices, USA) at 540 nm.

5.13. Immunostaining

5.13.1. VNPs on Spread Cells

hMSCs were labeled with Vybrant DiI (Thermo Fisher, USA) and seeded at 5000 cells per cm². The medium was changed on the next day to the VNP-laden medium at a concentration of 6 × 10⁻¹⁰ m VNP. After cells and VNP were cultured together for a day, cells were fixed with 4% v/v paraformaldehyde and rinsed with PBS three times. The cells were permeabilized with 0.1% v/v Triton X-100/PBS and washed with PBS three times, followed by incubation of 1% w/v BSA/PBS as blocking solution for 20 min. Each sample was labeled with anti-PVX antibody (DSMZ, 1:1000 in 1% w/v BSA/PBS) for an hour. Secondary antibody (AlexaFluor488, 1:2000 in 1% w/v BSA/PBS) was incubated with cells at RT for 45 min in the dark. The coverslips were mounted with ProLong mounting solution with DAPI (Thermo Fisher) and then imaged with fluorescent microscopy.

5.13.2. hMSCs in VNP-Laden Hydrogels

hMSCs were labeled with Vybrant DiD (Thermo Fisher) and encapsulated in 1% w/v agarose hydrogel with various VNP concentrations of 6 × 10⁻⁹, 6 × 10⁻¹⁰, 6 × 10⁻¹¹, and 6 × 10⁻¹² m. The hydrogel constructs were cultured in growth medium, which was changed three times per week and incubated at 37 °C. On days 0, 1, and 7, hydrogel constructs were washed with PBS and fixed with 4% v/v paraformaldehyde, followed by a PBS washing step. Afterward, hydrogel constructs were incubated in 0.1% v/v Triton X-100/PBS for 30 min and then in 3% w/v BSA/PBS for an hour at RT. VNP within the hydrogel constructs were stained with anti-PVX antibody (1:1000 in 3% w/v BSA/PBS) and secondary antibody (AlexaFluor488, 1:5000 in 3% w/v BSA/PBS) was incubated with cells at RT for 45 min in the dark. The hydrogel constructs were imaged by a TPLSM (FV1000MPE, Olympus, Japan) with a 25 × NA1.05 water immersion objective or a confocal laser scanning microscope (LSM 710, Zeiss, Germany) with a 40x /1.1 W Corr M27 objective.
5.14. VNP Release Rate

VNPs were encapsulated in 1% w/v agarose hydrogels at a concentration of \(6 \times 10^{-10}\) m. The hydrogels were immersed in PBS and incubated at 37 °C. The PBS was changed and collected every day for 5 days. ELISA was employed to quantify the amount of VNPs released in the collected PBS samples. To accomplish this, 50 µL PBS sample was coated with 50 µL coating buffer (CB, 15 mM Na2CO3, 35 mM NaHCO3, pH 9.6) or 100 µL of sample was coated directly, and WT PVX, PVX-HABP, and PVX-E8 in the range of 0.25–2.5 ng µL\(^{-1}\) were coated as standards for at least 2 h at 37 °C. The wells were blocked with 4% w/v skimmed milk in PBS overnight at 4 °C. As primary antibody, 100 µL anti-PVX antibody diluted 1:5000 in PBS was incubated for 2 h at 37 °C, followed by incubation of 100 µL for at least 2 h with an ALP-labeled secondary GAR antibody diluted 1:5000 in PBS. For detection, 200 µL p-nitrophenylphosphate (1 mg mL\(^{-1}\)) in substrate buffer (50 mM Tris/HCl pH 9.6, 150 mM NaCl, 2 mM MgCl\(_2\)) was used and the absorbance was measured after 60–90 min at 405 nm.

5.15. Mechanical Properties

5.15.1. Viscosity and Gelation Temperature Measurements

The viscosity and gelation temperature of the hydrogels were measured using a rotational rheometer (Kinexus pro+, Malvern Instruments, UK) with a 4° cone plate. For viscosity measurement, the temperature was set at 37 °C and the shear rate was increased from 0.1 to 1000 s\(^{-1}\) over a period of 30 min. The average shear viscosity values were calculated from the values with shear rates above 40 s\(^{-1}\). The gelation temperature was measured between 37 and 20 °C at a constant frequency of 1 Hz.

5.15.2. Mechanical Compression Test

VNP-laden hydrogels were cast in a cylindrical mold with a 15 mm diameter and a 10 mm height. An unconfined compression test was performed using a universal testing machine (MiniZwick, Zwick, Germany). The hydrogel constructs were compressed constantly between two impermeable metallic plates at a crosshead speed of 4 mm min\(^{-1}\) in a uniaxial direction. The compressive tangent moduli were determined from the slope of the stress-strain curve between 0–5%, 5–10%, 10–15%, and 15–20%.

5.16. Biomineralization, Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray Spectroscopy (EDX)

Both the VNP- and synthetic HABP-laden (Life Technologies, Germany) hydrogels were immersed in SBF\(^{[25]}\) and incubated at 37 °C for 21 days. After this, the hydrogel constructs were fixed in 3% v/v glutaraldehyde (Agar Scientific, Germany) in 0.1 M Sorensen’s phosphate buffer for at least 4 h at RT and washed with the same buffer for 15 min. They were then dehydrated with ascending ethanol series (30%, 50%, 70%, and 90% v/v; 10 min for each step) and finally 100% ethanol, 10 min three times. Subsequently, they were critical point dried in liquid CO\(_2\) (Critical Point Dryer, E3100, Quorum Technologies) and coated with a layer of carbon or 10 nm gold/palladium (Sputter Coater EM SCD500, Leica, Germany). Finally, the hydrogel constructs were investigated under an environmental scanning electron microscope (ESEM XL30 FEG, FEI, Netherlands) in a high vacuum with 10 kV acceleration voltage. The chemical composition of the samples was detected by an energy-dispersive X-ray spectroscopy (EDAX Genesis System).

5.17. Graphics and Software

Clone Manager Professional Suite 8 was used to calculate the molecular weights and IEPs of the proteins. Charts were designed using Microsoft Office Excel 2016.

5.18. Statistical Analysis

Data are presented as means ± standard deviations. The sample size number of all the experiments is \(n = 3\), except for VNP release rate (\(n = 4\)). Alizarin red staining was performed with two independent cell donors, while cell viability in VNP-laden hydrogels were performed with three. Each experiment from each cell donor contained sample size \(n = 3\). Data analyses of viscosity, gelling temperature, and compressive tangent modulus were performed using one-way analysis of variance followed by Bonferroni's post hoc test. Statistical significance was defined as *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.005\). Data analyses of all the other experiments were performed using Student’s t-test and statistical significance was marked as *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.005\).

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

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