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A bio-imitating approach to fabricate an artificial matrix for cartilage tissue engineering using magnesium-polyphosphate and hyaluronic acid

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Abstract

Here we describe an artificial cartilage-like material based on a hyaluronic acid-Mg/Ca-polyphosphate paste (HA-aMg/Ca-polyP-p) that is fabricated from the water-soluble Na-salt of energy-rich inorganic polyphosphate (polyP) and soluble hyaluronic acid in the presence of water-insoluble $CaCO_3$. The resulting material, after conversion of Na-polyP into the less soluble Mg/Ca-salt consisting of amorphous Mg/Ca-polyP microparticles, was found to mimic the physiological cartilage tissue and to bind Ca^{2+} ions present in the synovial fluid. After the Mg^{2+}/Ca^{2+} exchange and water extrusion the polyP becomes more stable, but is still susceptible to hydrolytic cleavage by the alkaline phosphatase (ALP). The material shows biomechanical properties, comparable to cartilage. Treatment with $CaCl_2$ resulted in an increase of the hardness (Young's modulus) from 1.27 MPa to 3.23 MPa. In addition, the $CaCl_2$-treated material showed a faster stress increase, almost no micro-crumbling and an extended creep period and elastic/viscoelastic recovery period. Moreover, the material exhibits morphogenetic activity through upregulation of the marker genes in chondrocytes encoding for ALP, collagen 2A1, collagen 3A1 and aggrecan. We propose that Mg-polyP is a promising material for cartilage repair that is able to scavenge, from the synovial fluid, Ca^{2+} ions implicated in crystal formation in osteoarthritis patients, as well as hyaluronic acid, through Ca^{2+}-bridge formation, and to act as a bonding material for cartilage and bone.

1. Introduction

Damage of cartilage is a relatively common type of injury, especially of the hyaline (articular) cartilage that covers adjacent bone surfaces of the joints where it functions to smooth surface during motion (reviewed in: ref.[1]). Often articular cartilage undergoes damage as a result of a direct injury, e.g., of the knee (reviewed in: ref.[2]). The consequences are swelling, stiffness, locking and limited movement. Since articular cartilage has only minimal capacity to repair damages it tends to generalize and to wear out resulting in osteoarthritis; in turn any damage to cartilage may initiate osteoarthritis formation. Surgical interventions have been proposed and successfully applied, e.g. osteochondral graft transfer,[3] microfractures,[4] cell-free scaffold-assisted one-step cartilage repair procedures[5] or autologous chondrocyte implantation.[6] However, considering the limitations of those treatments as well as the high costs and the need for several operations, like for autologous chondrocyte implantation, alternative approaches to repair cartilage have been tried out. Those new techniques involving the application of collagenic scaffold combined with microfractures[7] or synthetic polymers like polyglycolic acid together with hyaluronic acid have been shown to have the potential to restore hyaline-like cartilage.[8]

Recently we introduced a bio-imitating approach to prepare artificial cartilage implant material composed of the physiological polymer polyphosphate (polyP) that is abundantly present in certain blood cells, like platelets (reviewed in: ref.[9]) as well as in synovial fluid (to be published), and can be used as one multifaceted scaffold element for the fabrication of implants (reviewed in: refs.[10,11]). In addition, the likewise biocompatible natural polymers *N,O*-carboxymethyl chitosan and alginate has been added.[12] In order to avoid any non-physiological, foreign polymer we present now the formulation of an artificial cartilage implant material that is composed only of the physiological components, polyP and hyaluronic acid. The latter component, hyaluronic acid, has been successfully used in previous matrices for cartilage tissue, in gelatin/methacrylamide hydrogels.[13] In a previous study Wu et al.[14] have succeeded to conjugate polyP onto HA-adipic dihydrazide covalently and could demonstrate that the resulting hydrogel is enhancing tissue growth and provides constant osteoconductive stimulation to embedded murine osteoblast precursor cells. Furthermore those cells express the osteogenic marker gene alkaline phosphatase (ALP). The natural inorganic polymer polyP, as a polyanion, has the property to “cross-link” *via* ionic bonds with divalent cations, e.g. Ca^{2+} or Mg^{2+}.[15] During this process the material hardens. The hardness of the material can be achieved by varying the Ca^{2+} concentration or the duration of the exposure to this cation. In contrast to Ca-polyP, Mg-polyP is more soluble in aqueous milieu and has a strong affinity to chelex out Ca^{2+} ions from the environment and by that exchange Mg^{2+} by Ca^{2+} in the polyP salt (to be published). It should be emphasized that polyP is not only a suitable matrix polymer but also enhances the intracellular energy metabolism by increasing the number of mitochondria[16] and even elicits extracellularly biochemically usable energy during cleavage of the acid anhydride linkages by ALP. During this process metabolic fuel is certainly produced that is required for the maintenance of the extracellular structural and functional organization of the fibrillar network.[11]

In the present study we describe the formulation of an artificial cartilage material based on Mg-polyP that, together with hyaluronic acid, biomimics cartilage and comprises the advantageous property of the potential implant for an application *in vivo* to readily incorporate Ca^{2+}; this cation is abundantly present in the synovial fluid especially after cartilage damage and bone hydroxyapatite disintegration.[17] The new material shows biomechanical properties, comparable to cartilage, and elicits morphogenetic activity through upregulation of the genes in chondrocytes encoding for

ALP, *collagen 3A1 (COL3A1)* and *aggrecan*. The ALP is an essential enzyme in both hard and soft tissues, like in cartilage,[18] the fibrillar collagen 3A1 is likewise present in cartilage; its gene is induced in chondrocytes undergoing a healing process.[19] In addition to *collagen 3A1 (COL3A1)* expression studies we included also the response of the cells with respect to *collagen 2A1 (COL2A1)*. This type of collagen is highly expressed in cartilage.[20] Aggrecan is a large proteoglycan, framed by numerous chondroitin sulfate and keratan sulfate chains, that tightens articular cartilage and provides this tissue with the property to withstand compressive loads (reviewed in: ref.[21]). Since matrix metalloproteinases and aggrecanases play a critical role in aggrecan degradation these enzymes are associated with joint inflammation and overloading. Aggrecan gene expression has been found to be down-regulated in osteoarthritis cartilage.[22]

2. Material and methods

2.1. Materials

Sodium polyphosphate (Na-polyP of an average chain of 40 phosphate units) was obtained from Chemische Fabrik Budenheim (Budenheim; Germany). Hyaluronic acid (sodium salt from rooster comb - average weight of ≈1 x 10^6 Da; #H5388) was obtained from Sigma (Taufkirchen; Germany).

2.2. Preparation of hyaluronic acid-Mg/Ca-phosphate fluffy-microporous paste (HA-aMg/Ca-polyP-p)

The fabrication of hyaluronic acid-Mg/Ca-phosphate paste (HA-aMg/Ca-polyP-p) was performed according to the general description given previously for amorphous Ca-phosphate microparticles (aCa-polyP-MP).[16] In detail, 1 g of hyaluronic acid was dissolved in 10 ml of distilled water and supplemented with 1 g of solid Na-polyP. Subsequently, 0.5 g of solid $CaCO_3$ (#5616; Roth Karlsruhe; Germany) was added. During the reaction the pH was kept around 9, at the $CaCO_3$ concentration used here. To the resulting viscous hyaluronic acid/$CaCO_3$/polyP suspension 1.5 g of solid $MgCl_2 \cdot 6H_2O$ (#A537.1; Roth) in 5 ml of distilled water was added. Continuation of mixing allowed the formation of a white non-transparent paste, termed HA-aMg/Ca-polyP-p. After a 2 h period white flakes/deposits are formed.

In one series of experiments the hyaluronic acid component was omitted from the polyP/$CaCO_3$/$MgCl_2$ fabrication process; the resulting paste was termed aMg/Ca-polyP-p.

To investigate the change of the properties with respect to the exposure to Ca^{2+} the HA-aMg/Ca-polyP-p was incubated with 5 mM $CaCl_2$ for 3 d.

2.3. Microscopic inspections

Digital light microscopy was performed with a VHX-600 Digital Microscope (Keyence, Neu-Isenburg; Germany) equipped with a VH-Z100zoom lens. For the scanning electron microscopic (SEM) visualization a scanning electron microscope (Nova 600 Nanolab; FEI, Eindhoven; The Netherlands) was used.

2.4. X-ray diffraction, energy-dispersive X-ray, and Fourier transformed infrared spectroscopic analyses

X-ray diffraction (XRD) experiments were performed as described previously.[23] The patterns were registered on a Philips PW 1820 diffractometer with CuKα radiation (λ = 1.5418 Å, 40 kV, 30 mA) in the range 2θ = 5-65° ($\Delta 2\theta$ = 0.02, Δt = 5 s).

For energy-dispersive X-ray (EDX) spectroscopy an EDAX Genesis EDX System attached to the scanning electron microscope (Nova 600 Nanolab; FEI, Eindhoven, Netherlands) and operating at 10 kV with a collection time of 30-45 s was used.

Fourier transformed infrared spectroscopic (FTIR) analyses were performed with micro-milled powder in an ATR (attenuated total reflectance)-FTIR spectroscope/Varian 660-IR spectrometer (Agilent, Santa Clara, CA), fitted with a Golden Gate ATR unit (Specac, Orpington, UK), as outlined.[24] The spectral resolution of FTIR spectrometer was 4 cm^{-1}. For this series of experiments we used the paste formed from hyaluronic acid, Na-polyP and solid $MgCl_2$ as described above. No $CaCO_3$ was added to avoid any superimpositions; the hyaluronic acid-Mg-phosphate paste was termed HA-aMg-polyP-p.

2.5. Dissolution of HA-aMg-polyP-p by ALP

For the dissolution studies of 100 mg of either HA-aMg-polyP-p (or – as indicated – HA-aMg/Ca-polyP-p) or aCa-polyP-MP were suspended in 10 ml of 20 mM Tris-HCl buffer (pH 8.0). The aCa-polyP-MP particles were prepared as described.[16,25] Then recombinant bovine ALP, expressed in *Pichia pastoris* (#P8361; Sigma) was added at a final concentration of 20 units/ml. After an incubation period of 14 h or 48 h (at room temperature) the remaining pellet was analyzed by eye inspection and FTIR.

2.6. Human chondrocytes

Human chondrocytes were obtained from (Lonza GmbH, Köln, Germany; NHAC-kn, #CC-2550). Those primary cells were used for the studies. They were cultivated in Chondrocyte Growth Medium (CGM BulletKit; Lonza #CC-3216) which contains growth factors and 5% fetal bovine serum (FBS). The cells were subcultured after reaching 80%-90% confluency. They were seeded with a density of about 10000 cells per cm^2; cell number duplicates about every 60 hrs. The Chondrocyte Growth Medium contains ≈2 mM of Ca^{2+} and ≈1mM of Mg^{2+}. Applying the Spectroquant-test kit (Millipore-Merck, Darmstadt; Germany) as well as the Reflectoquant system (Millipore-Merck) for determining of Ca^{2+} as well as of Mg^{2+} in the medium it could be verified that the concentration of these two ions did not change significantly during the 21 d incubation period.

For the cell growth as well as the qRT-PCR experiments the cultures were incubated for 6 d or 12 d/21 d, respectively. The paste or the particles do not float on the surface of the culture medium but sediment to the bottom of the well plates. In turn, after seeding of the cells, they readily attach to the surface of the biomaterial and start to grow as monolayer.

To determine the growth rate of the cells, the chondrocytes were seeded into 6-well plates and cultured for 6 d in medium/serum. The following components were added separately to the cultures; 100 µg/ml of hyaluronic acid or 50 µg/ml either of Na-polyP (complexed stoichiometrically with Ca^{2+} [molar ratio of 2 phosphate monomers to 1 Ca^{2+}][26], aMg/Ca-polyP-p, or of HA-aMg/Ca-polyP-p. After termination the cells were incubated with 3-[4,5-dimethyl thiazole-2-yl]-2,5-diphenyl tetrazolium (MTT; #M2128, Sigma) as described.[25] Subsequently the remaining MTT dye was aspirated and 200 ml of DMSO were added to solubilize the formazan crystals. Finally, the optical densities (OD) were determined at 650 nm. Ten parallel experiments each were performed. After seeding the MTT assay, the viability both of the fee-floating and the cells, attached to the biomaterial, had been measured. Since the viability studies had been performed 60 min (latest) after seeding the cells into the culture medium no differences between the different assays, supplemented with the different biomaterials, could been measured.

In one series of experiments, chondrocyte layers were stained with haematoxylin-eosin stain (Sigma), as described.[27]

2.7. Gene expression studies

The technique of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was applied to quantitate semiquantitatively the effect of the polyP samples on chondrocytes. The cells were incubated for 12 d or 21 d either in the absence of any additional component besides of medium/serum, or in the presence of 0.5 mg/ml of hyaluronic acid, 50 µg/ml of Na-polyP (complexed with Ca^{2+} at a molar ratio of 2 [with respect to phosphate monomer] to 1 [Ca^{2+}]),[26] 50 µg/ml of aMg/Ca-polyP-p or of HA-aMg/Ca-polyP-p. Technical details have been given before.[28] The following four genes were selected to determine the activation state of the human chondrocytes in culture; *ALP* (alkaline phosphatase; NM_000478.4) Fwd: 5′-TGCAGTACGAGCTGAACAGGAACA-3′ (nt_{1141} to nt_{1164}) and Rev: 5′-TCCAC

CAAATGTGAAGACGTGGGA-3′ (nt_{1418} to nt_{1395}; 278 bp), *COL2A1* (collagen type II alpha 1; NM_001844) Fwd: 5′-TCCATTCATCCCACCCTCTCAC-3′ (nt_{4755} to nt_{4776}) and Rev: 5′-TTTCCTGCCTCTGCCTTGACC-3′ (nt_{4902} to nt_{4882}; 148 bp); *COL3A1* (collagen type III alpha 1; NM_000090) with the primer pair Fwd: 5′-ATTCCTTCGACTTCTCTCCAGCC-3′ (nt_{4182} to nt_{4204}) and Rev: 5′-GTGTTTCGTGCAACCATCCTCC-3′ (nt_{4377} to nt_{4356}; 196 bp) as well as *ACAN* (aggrecan transcript variant 1; NM_001135.3) Fwd: 5′-CCTCTGCATTCCACGAAGCTAACC-3′ (nt_{6772} to nt_{6795}) and Rev: 5′-TGCCTCTGTCCCCACATCACTG-3′ (nt_{6917} to nt_{6896}; 146 bp). The expression levels of these transcripts were correlated to the reference housekeeping gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase, NM_002046.3) Fwd: 5'-ACTTTGTGAAGCTCATTTCCTGGTA-3' (nt_{1019} to nt_{1043}) and Rev: 5'-TTGCTGGGGCTGGTGGTCCA-3' (nt_{1136} to nt_{1117}; 118 bp). The quantitative real-time PCR experiments were performed in an iCycler (Bio-Rad); the mean C_t values and efficiencies were calculated with the iCycler software (Bio-Rad, Hercules, CA, USA); the estimated PCR efficiencies range between 93% and 103%.[29]

2.8. Determination of the biomechanical properties of the material

The overall biomechanical properties (the bulk Young's modulus) of the Mg-polyP-based cartilage scaffolds were determined using standardized cylindrical material samples measuring 5.4 mm in diameter and 4-5 mm in length, using the "MultiTest 2.5-xt Force Testing System" fitted with a 100 N Load Cell Unit (Mecmesin Ltd., Slinfold; UK). The data was recorded at a continuous recording frequency of 50 Hz using the Emperor XT Force software (Mecmesin Ltd.). A force of 0.5 N was found to be suitable to determine the unconfined compressive strength; during the measurements it was kept constant for 60 s. Subsequently, an unloading period (0 N) followed for 300 s. From the force and displacement data obtained, the bulk Young's modulus was calculated. The following equation was used: E [Young's modulus] equals to σ [stress]/ε [strain], whereby σ = force (N)/area (mm^2) and ε = ΔL [difference in length] (mm)/L_0 [initial length] (mm).

For the recording of the creep-recovery-curves a constant load of 2.5 N was applied to the samples and held for 1800 s. Subsequently, the recovery period at 0 N followed which was limited to 600 s. The data for the creep-recovery-curves were recorded at a frequency of 50 Hz and again used to calculate the strain (ε) as described[30] for the preparation of the strain-time graphs.

2.9. Water content

The two scaffold forms, HA-aMg/Ca-polyP-p and HA-aMg/Ca-polyP-p-treated with 5 mM $CaCl_2$ for 3 d, were cut to slices to approximately the same dimensions. After removal of the superficial, oozing liquid the samples were freeze-dried overnight (Sentry 2.0 lyophiliser; SP Scientific, Warminster; PA). Subsequently, the dry weight was determined and the % water content was calculated.

2.10. Statistical analysis

After finding that the respective values follow a standard normal Gaussian distribution and that the variances of the respective groups are equal, the results were statistically evaluated using the independent two-sample Student's *t*-test.[31]

3. Results

3.1. Fabrication of hyaluronic acid-Mg/Ca-phosphate fluffy-microporous paste (HA-aMg/Ca-polyP-p)

The fabrication of hyaluronic acid-Mg/Ca-phosphate paste (HA-aMg/Ca-polyP-p) was performed by successive addition of solid, water-soluble Na-polyP to soluble hyaluronic acid, as described under “Materials and Methods”. Then solid $CaCO_3$ was added which did not dissolve in the viscous hyaluronic acid/polyP solution (Fig. 1A and B). After addition of solid $MgCl_2$ the paste started to dissolve at the rim under formation of a 500 µm sized fibrillar net by ionic gelation (Fig. 1C). This series of steps was microscopically followed in a one-cavity microscope slide. Eye-inspection of these steps in 1.5 ml Eppendorf tubes revealed that the particles, composed of hyaluronic acid/Na-polyP/$CaCO_3$ did not sediment under normal conditions (Fig. 1D). After addition of $MgCl_2$, paste-like deposits are formed that sediment to the bottom of the tubes by forming the HA-aMg/Ca-polyP-p paste (Fig. 1E and F).

The microscopic morphology of the paste, HA-aMg/Ca-polyP-p, was inspected by digital light microscopy as well as by SEM (Fig. 2). At lower magnification, and before freeze-drying, the HA-aMg/Ca-polyP-p appeared as a fluffy scaffold (Fig. 2A) with a shed-like lattice (Fig. 2B). Visualization by SEM revealed globular particles ranging between 320 and 560 nm (Fig. 2C). After freeze-drying the fluffy scaffold turns to a more solid deposit (Fig. 2D). After addition of 5 mM $CaCl_2$ to HA-aMg/Ca-polyP-p (3 d) the material became more compact, as can be deduced before (Fig. 2E; surface morphology) and after freeze-drying (Fig. 2F; surface).

XRD analysis of the freeze-dried HA-aMg/Ca-polyP-p revealed no peak between a 2-theta range 5-50 degree (data not shown), indicating that the globular particles in HA-aMg/Ca-polyP-p are amorphous, as previously described for the Ca-polyP microparticles (aCa-polyP-MP).[16]

For the analysis of the elements, present in the HA-aMg/Ca-polyP-p, EDX spectroscopy was applied. As shown in Fig. 3, the spectrum shows the characteristic signals for O, P, Na (remaining from the Na-polyP starting material) as well as of Mg and Ca both linked *via* ionic bonds to the polyP backbone, under formation of insoluble deposits. The element weight-% ratio, calculated from the EDX data analyses, was determined to be Ca:Na:Mg with 1:0.22:0.14.

3.2. Dissolution of HA-aMg/Ca-polyP-p by ALP

In a comparative study aCa-polyP-MP *versus* HA-aMg/Ca-polyP-p the materials were incubated with ALP, as described under “Materials and Methods”. Even by eye inspection it becomes obvious that during the 48 h incubation period the aCa-polyP-MP particles undergo only a lower hydrolytic dissolution, if compared with HA-aMg/Ca-polyP-p (Fig. 4). During this incubation period only about 48% (by dry weight) of the aCa-polyP-MP dissolved were transferred to a soluble form (Fig. 4; lanes a and b). In contrast, (almost) the total amount of HA-aMg/Ca-polyP-p was solubilized in the presence of ALP (lanes c and d).

In order to analyze also the process of hydrolysis of HA-aMg-polyP-p (due to technical reasons this paste was used instead of HA-aMg/Ca-polyP-p) also qualitatively the sediments were dried and subjected to FTIR spectral analysis (Fig. 5). The HA-aMg-polyP-p were either used directly for the analysis or were incubated in Tris-HCl buffer in the absence or presence of ALP for 14 h. The results of the non-incubated material show the main characteristic peaks for HA-aMg-polyP-p material; the peaks at the wavenumbers 1240 cm^{-1} (symmetric stretching mode $\nu_s(PO_2)^{2-}$), 1103 cm^{-1} ($\nu_{as}(PO_3)^{2-}$), 897 cm^{-1} (ν_s(P-O-P)) and 723 cm^{-1} (ν_{as}(P-O-P)), which indicate the

polyphosphate microstructure (Fig. 5). Those peaks remained almost unchanged during a 14 h incubation period in Tris-HCl buffer. However, after addition of ALP a significant shift of the bands occurred. Especially pronounced are the following shifts; increase of the absorbance bands in the region of 1100 cm^{-1} to 980 cm^{-1} due to P-O stretching vibrations from PO_4^{3-} vibrational mode of the phosphate group. While the polyphosphate reflected by the band at 1240 cm^{-1} disappeared and the intensity of peak at 897 cm^{-1} was decreased and split into the two peaks at 913 cm^{-1} and at 893 cm^{-1}, which is characteristic of the P-O stretching vibrations from PO_4^{3-} groups. The above results indicate that the HA-aMg-polyP-p have undergone hydrolytic degradation, induced by ALP.

3.3. Morphology

For the required mechanical studies cylindrical blocks (5.4 mm [diameter] x 4-5 mm [length] were prepared (Fig. 6A). The surface of those samples was porous with an average pore size of ≈15 µm (Fig. 6B). Breaking the blocks revealed that the interior of the samples is traversed by larger channels with an average dimension of ≈25 µm (Fig. 6C).

3.4. Determination of the biomechanical properties of the material

Compression tests were performed using a tensile/compression test system to characterize the bulk mechanical properties of the polyP material. In order to study the effect of Ca^{2+} on the hardness/consistency of HA-aMg/Ca-polyP-p the material was incubated in the presence of 5 mM $CaCl_2$ for 3 d at ambient temperature.

Already the shapes of the stress-strain curves (Fig. 7) indicate the change in the properties of HA-aMg/Ca-polyP-p after exposure to Ca^{2+}. In the absence of the cation the polymer initially shows a prolonged deformation segment upon loading which also comprises discontinuities that reflect micro-crumblings of the material. After reaching the maximal stress of 0.025 MPa the loading stress was kept constant and a creep phase could be observed which is terminated with the beginning of the unloading phase. In the following the stress was kept constant at 0 MPa. The pronounced indentation to the left shows a partial recovery of the material and thus indicates some elastic/viscoelastic properties of HA-aMg/Ca-polyP-p. The Young's modulus which was calculated at the end of the loading phase amounts to 1.27 MPa.

In contrast to HA-aMg/Ca-polyP-p the material exposed to $CaCl_2$ shows a faster stress increase which is reflected by a higher slope of the initial deformation. The increase of the curve is close to perfect linear indicating that no distinct micro-crumblings occur (Fig. 7). The creep period is longer if compared to HA-aMg/Ca-polyP-p and likewise the final elastic/viscoelastic recovery period is also extended. The Young's modulus – as expected – is considerably numerically higher and measures 3.23 MPa. This shift in the mechanical properties in response to Ca^{2+} is seen already after an exposure time of 60 min to $CaCl_2$. For those experiments, we have used the maximal stress force that the material, fabricated here, is allowed to apply. By that we measured at day 1 a Young's modulus of 1.27 MPa and after 3 day a value of 3.23 MPa. Those values had been measured also previously for native (porcine) cartilage.[12] These differences between the material prior and after treatment with $CaCl_2$ is also reflected by the creep measurements using constant compressive stress for 30 min followed by a 10 min recovery time (Fig. 8). The untreated HA-aMg/Ca-polyP-p shows a similar biphasic creep-recovery curve with incomplete recovery upon unloading compared to the material treated with $CaCl_2$. However, the Ca^{2+}-treated material shows a more pronounced creep behavior. The initial strain response is faster and already reaches the maximal strain response after ≈ 5 s, with ≈ 0.23 mm/mm. Both samples

were loaded with a force of 2.5 N. In a further series of experiments we extended the creep measuring time to 30 min; no significant differences have been determined (not shown here).

The water content of the unprocessed HA-aMg/Ca-polyP-p was found to be 77.33±1.64%; in comparison the $CaCl_2$-treated samples contained slightly less water with 63.38±0.78%. Ten independent experiments were performed.

3.5. Bio-compatibility of the surface to the HA-aMg/Ca-polyP-p *material*

If HA-aMg/Ca-polyP-p past pieces are formed by ionic gelation of the polymers (Fig. 9A) and put into a chondrocyte culture in medium/serum for 24 h a homogenous cell layer is formed on their surfaces (Fig. 9B). The cell layer was visualized by haematoxylin-eosin color reaction.

For cell culture studies with the aim to assess the effect of the biomaterials, used in the present study, on cell growth (metabolic activity), the MTT assay was applied. The cells were cultivated either in the absence (control) or presence of the following components; 100 µg/ml hyaluronic acid, or of 50 µg/ml either of Na-polyP, aMg/Ca-polyP-p, or of HA-aMg/Ca-polyP-p (Fig. 9C). At time zero (seeding the cells) the absorbance at 650 nm was measured with 0.82±0.11 absorbance units. Incubating the cells in the absence of any additional component (controls) the metabolic activity drops significantly to 0.63±0.08 units, while all other assays which contained hyaluronic acid and/or polyP show a significant increase in the activity. The highest stimulation of growth was determined in the assays with HA-aMg/Ca-polyP-p (1.41±0.19) and aMg/Ca-polyP-p (1.28±0.16); the values are significantly higher if compared with the values measured for hyaluronic acid (0.93±0.13).

3.6. Expression of the genes encoding for ALP, collagen type III alpha 1 and aggrecan in chondrocytes

The steady-state-expression of four determining genes for chondrocytes, *ALP*, *collagen type II alpha 1* (*COL2A1*), *collagen type III alpha 1* (*COL3A1*) and *aggrecan* was selected for the determination of the morphogenetic activity using the qRT-PCR technique; the house-keeping gene *GAPDH* was used as a reference gene. Cells were incubated for 12 d or 21 d without any additional component (controls) or with 0.5 mg/ml of hyaluronic acid or 50 µg/ml either of Na-polyP, aMg/Ca-polyP-p or of HA-aMg/Ca-polyP-p (Fig. 10). The response of the *ALP* gene expression was rapid and significant for all three polyP containing samples, already after the 12 d incubation (Fig. 10A). The highest *ALP* expression levels were determined after 21 d for aMg/Ca-polyP-p (3.2-fold induction compared to the controls after the same incubation period) and HA-aMg/Ca-polyP-p (3.9-fold).

The expression of *COL2A1,* the major fibrillar molecule in cartilage, is significantly upregulated in those chondrocytes that are exposed for 12 d to the biomaterial composed of polyP, HA and HA-aMg/Ca-polyP-p (1.6-fold). After a prolonged incubation period of 21 d both the HA-free [aMg/Ca-polyP-p] and HA-containing artificial cartilage material [HA-aMg/Ca-polyP-p] elicit a significant increased in the steady-state-expression level by 1.7- and 2.2-fold, respectively (Fig. 10B).

The expression profile for *COL3A1* is very much similar than the one measured in the *ALP* assays. Again the highest expression levels are seen after 21 d with 2.9-fold in the presence of aMg/Ca-polyP-p, and 3.8-fold for HA-aMg/Ca-polyP-p (Fig. 10C) under the experimental conditions used here, again no significant stimulatory effect was measured for hyaluronic acid.

The expression level of the *aggrecan* gene was lower in chondrocytes compared to the ones of *ALP, COL2A1* and *COL3A1*. However, also using this gene as a marker

the expression in assays with aMg/Ca-polyP-p and HA-aMg/Ca-polyP-p were significantly upregulated at both incubation time periods (Fig. 10D). Again the highest expression level was measured for HA-aMg/Ca-polyP-p (2.1-fold) after the 21 d incubation.

4. Discussion

In general it appears that cartilage, due to its low cellularity and avascular matrix, might be a suitable and promising candidate for tissue engineering.[32] In addition, this tissue seems to have a lower demand for metabolite transport and in turn elicits a lower risk of implant rejection *via* the immune system. However, biochemical considerations let us to convince that the structural and functional organization of the voluminous cartilage scaffold, surrounding the few chondrocytes, requires biologically usable energy to maintain their organized architecture. Until now, the only extracellular metabolic fuel which might function as an energy source to maintain the organization is polyP (reviewed in: refs.[11,16]). It has been described that polyP, added to chondrocytes *in vitro* induces both glycosaminoglycan and collagen synthesis in a concentration- and chain-length-dependent manner.[33] However, the soluble, unprocessed polyP might not be suitable for inclusion in an artificial cartilage due to its propensity to be readily cleaved hydrolytically by the ALP. The highly soluble Na-polyP can be converted to the less soluble, related Mg^{2+} and Ca^{2+} salts in a controlled manner under formation of sub-microparticles that are amorphous;[16] the aqueous solubility of polyP decreases gradually from Na^{+} *via* Mg^{2+} to Ca^{2+}.[34] Those particles have been found to display morphogenetic activity both *in vitro*[12] and *in vivo*[35].

The synovial fluid surrounding the articular cartilage contains by far more Ca^{2+} (136 mEq•l^{-1}), compared to Na^{+} (4 mEq•l^{-1}), or Mg^{2+} (4 mEq•l^{-1}).[36] Based on this relationship we fabricated Mg-polyP with the rational to act as a “scavenger” for free Ca^{2+}. An increased Ca^{2+} level in the synovial fluid has been implicated in the etiology of the calcium phosphate crystal formation.[36] Those crystals are frequently found in osteoarthritis patients and are considered the cause of cartilage damage.[37] Hence a lowering of the Ca^{2+} level of the synovial fluid might be beneficial for the amelioration of osteoarthritis and cartilage loss.[38] Therefore, we fabricated in the present study Mg-polyP with the goal to use this polyP polymer in a future application study as a scaffold for drawing out free Ca^{2+} from the synovial fluid (see scheme in Fig. 11). Mg-polyP can be prepared as amorphous microparticles (submitted). Since Mg-polyP is readily dissolved during a 2 d-incubation period in culture medium/serum it can be presumed that also in the synovial fluid the ionic bonds are released in the aqueous environment and allow the cation Ca^{2+} to bind to the polymer; after this cation exchange reaction the polymer is more stable in the aqueous milieu, but still prone to ALP hydrolysis.[16] During this process, the Mg^{2+}/water extrusion and Ca^{2+} bridge formation between the anionic polymer, its anionic groups are transiently accessible also for the formation of a second Ca^{2+}-bridge formation to hyaluronic acid. This anionic polymer abundantly exists in the synovial fluid with a level of about 2 mg/ml.[39] Therefore, it can be anticipated that the injection of Mg-polyP microparticles into the synovial fluid will be rebuilt to HA-Ca-polyP – surely amorphous – microparticles (Fig. 11). At present, since no *in vivo* data are available, the extent of Ca^{2+} uptake into the Mg^{2+}-polyP particles or pastes cannot be given. Future studies are required, especially after performing animals experiments, to answer the question on the dynamics and duration of the Mg^{2+} to Ca^{2+} exchange process. In a recent study we could demonstrate that the human osteoblast-like SaOS-2 cells infiltrate in the *N,O*-CMC [*N,O* carboxymethyl chitosan], polyP and alginate, the artificial cartilage biopolymers, after an incubation period of 2d.[12] In line with these results we presume that the chondrocytes, used in the present study, have a similar capacity. This finding implies that these cells express and release the ALP that dissolves the main component of the artificial cartilage, polyP. At present it is difficult to predict how long the core implant will resist a complete dissolution *in vivo*. Since the synovial fluid contains polyP (to be published) and also Ca^{2+}, especially in the fluid of patients with osteoarthritis[40] and based on previous *in vitro* findings[16], we assume that

the inorganic polymer remains longer than 2 weeks on the impaired place before it will be replaced by the subsequently formed HA-based hydrogel.

The biomaterials, fabricated in the present study, are not toxic to the cells. This conclusion has been drawn from the findings that the cells in the different assays show proliferation after seeing and also show an enhanced spreading phenotype. At the present time it cannot be decided if the polyP-based biomaterial displays its advantageous potential to the cells *via* direct cell-solid material contact or after dissolution and diffusion.

To rebuild this process, polyP as a Ca^{2+}-binding polymer as well as a metabolic fuel source, we fabricated Mg-polyP together with hyaluronic acid, in the presence of small amount of the highly water-insoluble $CaCO_3$ (solubility at 25°C of 13 mg/L). With time – after about 20 min – the exchange reaction Mg^{2+} by Ca^{2+} was terminated (HA-aMg/Ca-polyP-p). The resulting material had a consistency of a paste and was found to consist of 77% water, a value that matches the content in human cartilage.[41] In order to demonstrate the Ca^{2+}-capturing property of HA-aMg/Ca-polyP-p the fabricated material was exposed to 5 mM $CaCl_2$. Biomechanical determinations revealed that the hardness/Young's modulus increased from 1.27 MPa to 3.23 MPa, after treatment with $CaCl_2$. Parallel with this change the $CaCl_2$-treated material shows a faster stress increase and an almost complete lack of micro-crumblings. In addition, the creep period extends and the final elastic/viscoelastic recovery period extends as well. Those advantageous properties of the artificial cartilage material are also corroborated by the finding that the water concentration decreases only to 63%, a value that meets physiological values.

Importantly, the HA/Mg-polyP material fabricated here is prone to enzymatic hydrolysis in response to ALP. This process could be exemplarily recorded by FTIR spectroscopy, focusing on the peak with a wavenumbers 1240 cm^{-1} ($\nu_s(PO_2)^{2-}$ in polyP) which during enzymatic hydrolysis disappeared, while the intensity of peak at 897 cm^{-1} (ν_s(P-O-P)) was decreased and concurrently split into the two peaks at 913 cm^{-1} and at 893 cm^{-1}. This finding is indicative that also the metabolic energy which is stored in the phosphoanhydride bonds of polyP and liberated after hydrolysis of this linkage is released.

Supportive for the mentioned and released metabolic energy is also the finding that Mg-polyP, especially if linked with HA/Mg-polyP elicits proliferation-inducing activity; even more the polymer elicits morphogenetic activity as being deduced from the steady-state-expression studies using the marker genes *ALP*, *collagen 2A1* (*COL2A1*)*, collagen 3A1* (*COL3A1*) and *aggrecan*. An increased expression of collagen is not restricted to *COL2A1* only, the dominant collagen type during wound healing[42], but also to collagen type *COL3A1*, that type which has been determined to be upregulated during cartilage repair *in vivo*.[43] It is important to note that the expression of *COL2A1* is not reduced but enhanced, reflecting the positive regulatory potential on chondrogenesis of the biomaterial developed here. The *ALP* is especially expressed to a high levels on the surface of cartilage,[44] while a positive gene expression levels of the fibrillar *collagen 2A1* and *collagen 3A1* is indicative for an increased proliferation and differentiation state of chondrocytes.[45] The increased *aggrecan* gene expression, in response especially to growth onto HA-aMg/Ca-polyP-p, might indicate to a potential rescue effect for the impaired cartilage-specific proteoglycan core in osteoarthritis cartilage.[22]

5. Conclusion

The data presented in this study show that polyP, in the salt-form of Mg-polyP, is a promising biomaterial which comprises similar biomechanical properties like

physiological cartilage. This material can be fabricated together with hyaluronic acid and in the presence of Ca^{2+} to a scaffold that shares with physiological cartilage the property to allow chondrocytes to settle and to stimulate them to a higher proliferation state. Moreover, the material can be packed together with hyaluronic acid to a hybrid material that elicits morphogenetic activity. In future studies we plan to inject in animal studies Mg-polyP, without Ca^{2+} or $CaCO_3$, into the synovial fluid to verify that these microparticles are able to take up Ca^{2+} from the synovial fluid and incorporate them into the biomaterial under the release of Mg^{2+}. During this process the opening of the ionic bonds in Mg-polyP should allow in this transient state not only to form Ca^{2+}-salt bridges between two polyP chains but also to the anionic polymer hyaluronic acid.

Since the Mg-polyP-based HA-aMg/Ca-polyP-p paste is enzymatically dissolvable and elicits morphogenetic signals it has the potential to become a genuine regeneratively active artificial cartilage material.

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Fig. 1. Hyaluronic acid-Mg/Ca-phosphate fluffy-microporous paste (HA-aMg/Ca-polyP-p). (**A**, **B** and **D**) A soluble hyaluronic acid/Na-polyP solution was prepared to which solid $CaCO_3$ was added (HA/polyP/$CaCO_3$). The suspension was pipetted onto a concave microscope slides (A and B). After addition of $MgCl_2$ (**C** and **E**, **F**) a fibrillar net (arrow to fn in C) starts to be formed at the rim of the suspension, reflecting the dissolution of the solid $CaCO_3$ salt particles. Simultaneously a paste is formed composed of hyaluronic acid, polyP, Mg^{2+} and Ca^{2+} (HA-aMg/Ca-polyP-p). Prior to the addition of $MgCl_2$ to HA/polyP/$CaCO_3$ (D) the particles are homogenously distributed. (E and F) After supplementation with of $MgCl_2$ phase separation proceeds; the clear supernatant (composed primarily of NaCl fluid; sup) progressively increases and allows the HA-aMg/Ca-polyP-p particles to sediment (< >).

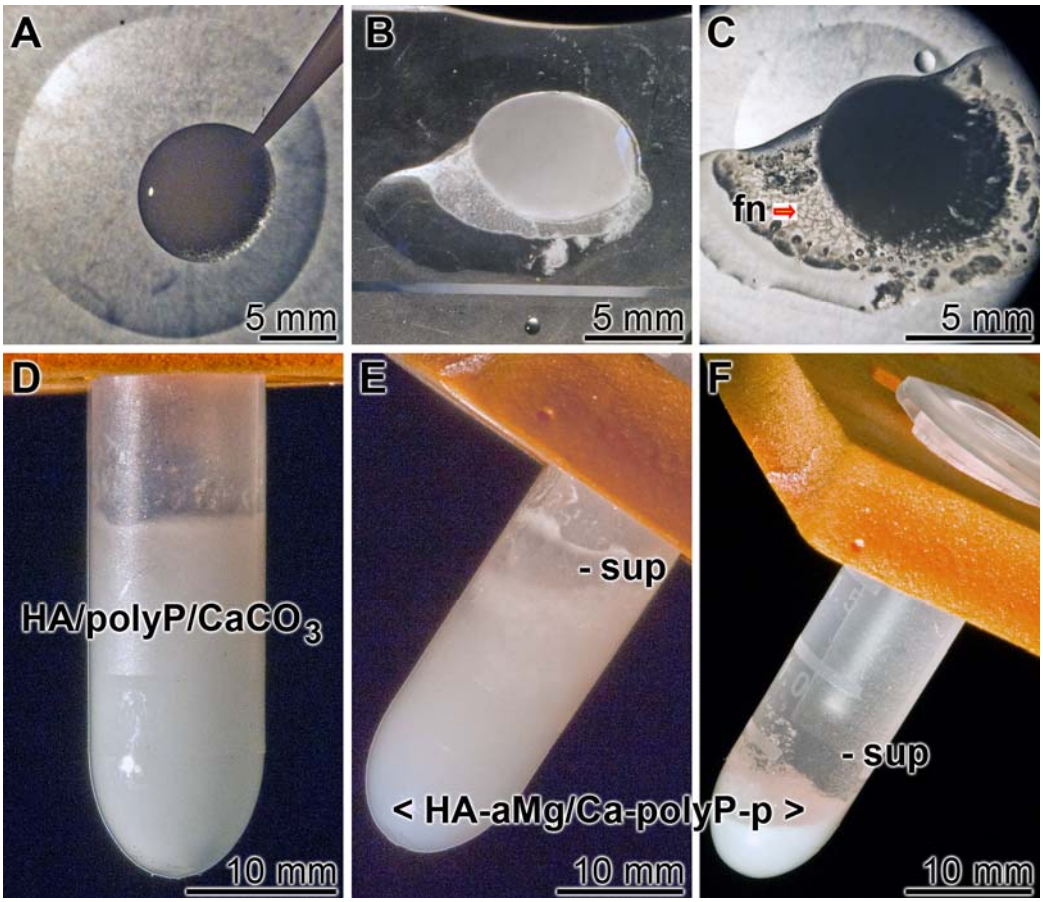

Fig. 2. Surface morphology of the HA-aMg/Ca-polyP-p paste. (**A** and **B**, and **D**-**F**) Digital light microscopic inspection of the HA-aMg/Ca-polyP-p prior to (A and B) and after freeze-drying (D). In a separate series of experiments 5 mM $CaCl_2$ was added to HA-aMg/Ca-polyP-p; view before (E) and after freeze-drying (F). (**C**) SEM image showing the globular particles in HA-aMg/Ca-polyP-p that are glued together.

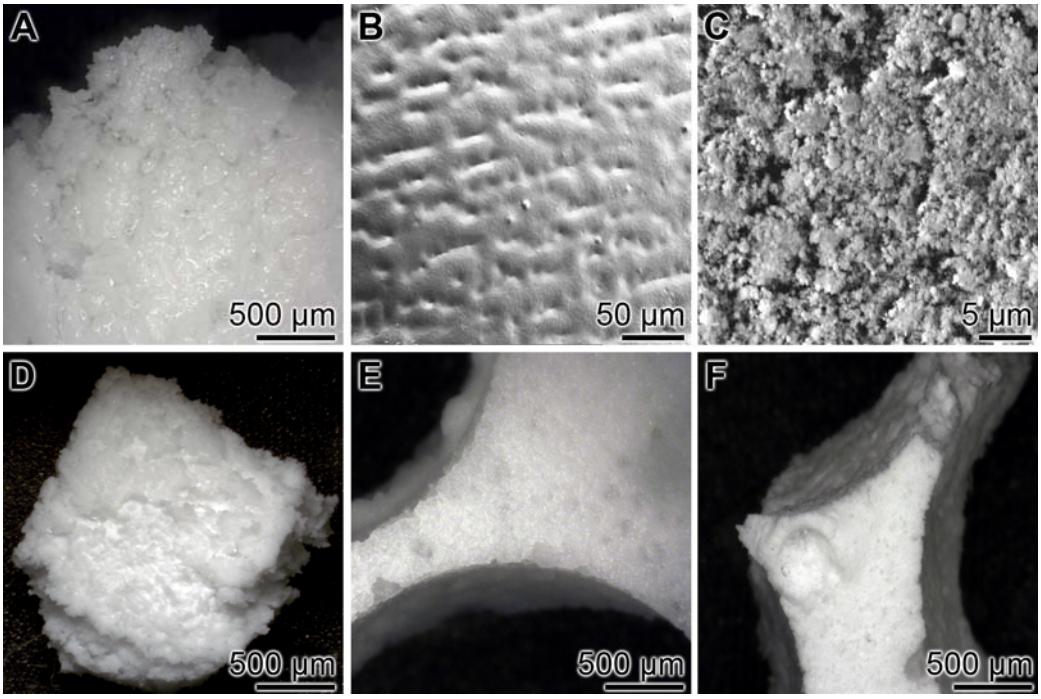

Fig. 3. EDX analysis of HA-aMg/Ca-polyP-p. The prominent element peaks (O, Na, Mg, P and Ca) are marked.

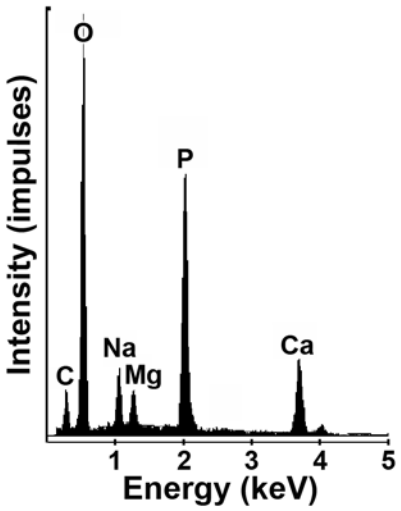

Fig. 4. Dissolution study of aCa-polyP-MP (**lanes a** and **b**) and of HA-aMg/Ca-polyP-p (**lanes c** and **d**). The microparticles were dissolved in Tris-HCl buffer (pH 8.0) and incubated for 0 h (lanes a and c) or 48 h (lanes b and d) in the presence of ALP.

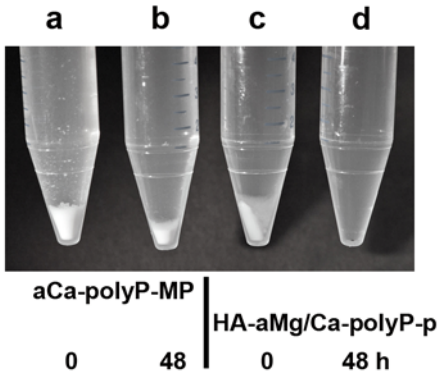

Fig. 5. Enzymatic hydrolysis of HA-aMg-polyP-p by ALP, as determined by FTIR spectroscopy. The spectra before (HA-aMg-polyP-p) and after transfer to Tris-HCl buffer (HA-aMg-polyP-p Tris) were determined. Finally, the FTIR spectrum was recorded after a 14 h incubation period in the presence of the ALP (HA-aMg-polyP-p ALP/Tris), as described under “Results”.

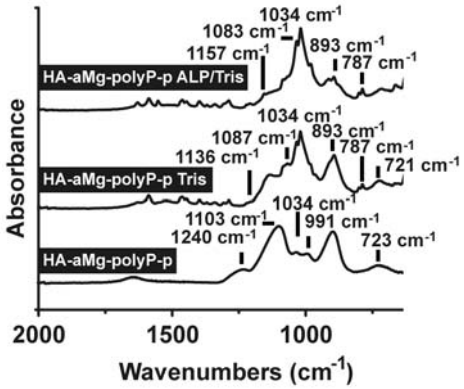

Fig. 6. Cylindrical blocks were prepared for subsequent biomechanical studies. (**A**) One cylinder is depicted. The light microscopic inspection revealed that the surfaces of the cylinders (**B**) comprise smaller channel pores (≈15 µm) compared to those that are found in the internal blocks with ≈25 µm (**C**).

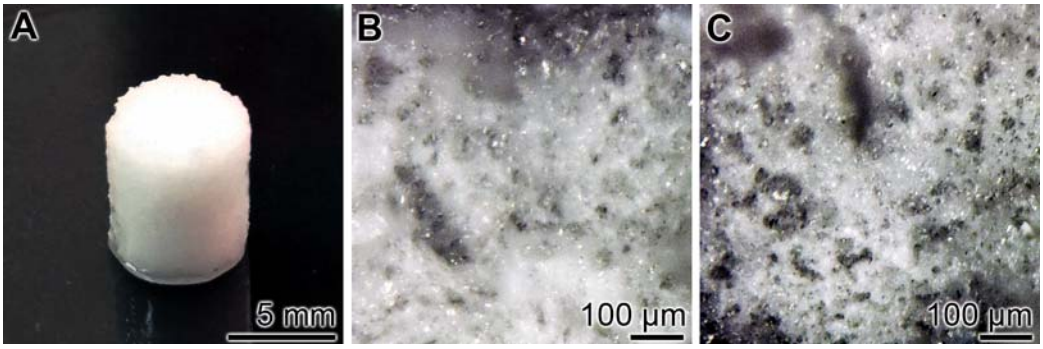

Fig. 7. Stress-strain curves for the HA-aMg/Ca-polyP-p scaffolds before (------) and after incubation with 5 mM $CaCl_2$ for 3 d (- - - -).

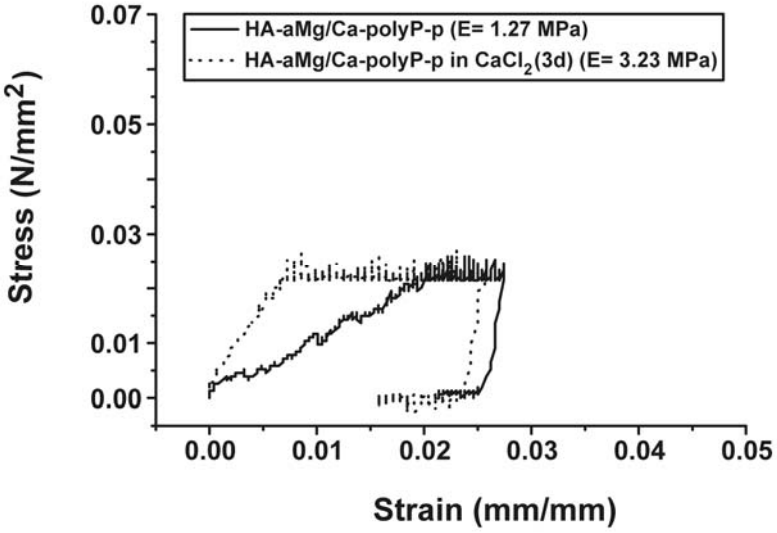

Fig. 8. Creep behavior of the HA-aMg/Ca-polyP-p material prior (------) and after a 5 mM $CaCl_2$ incubation for 3 d (- - - -).

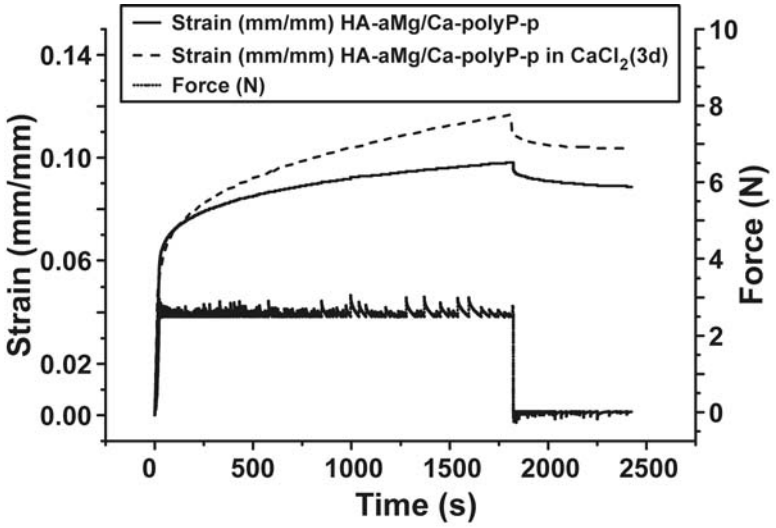

Fig. 9. Cell metabolic activity/growth of chondrocytes onto the biomaterials, described here. (**A**) One piece of HA-aMg/Ca-polyP-p in the absence of cells. (**B**) A HA-aMg/Ca-polyP-p layer was placed into a chondrocyte medium/serum suspension and incubated for 24 h. Then the sample was removed and stained with haematoxylin-eosin stains; a homogenous cell layer (**c**) appears. (**C**) MTT assay result of the viability/growth studies with the additional components after cultivation for 6 d in the absence of any additional component (control; open bars) or in the presence of hyaluronic acid (hatched to the right), Na-polyP (hatched to the left), aMg/Ca-polyP-p (cross-hatched) and HA-aMg/Ca-polyP-p (filled). The absorbance value at time zero is likewise given (grey bar). Standard errors of the means (SEM) are indicated (n = 10 experiments per time point; 5 parallel series of experiments). * $P < 0.05$, with respect to the values of the controls after the 6 d incubation period; § $P < 0.05$ compared to the viability of the cells incubated with hyaluronic acid.

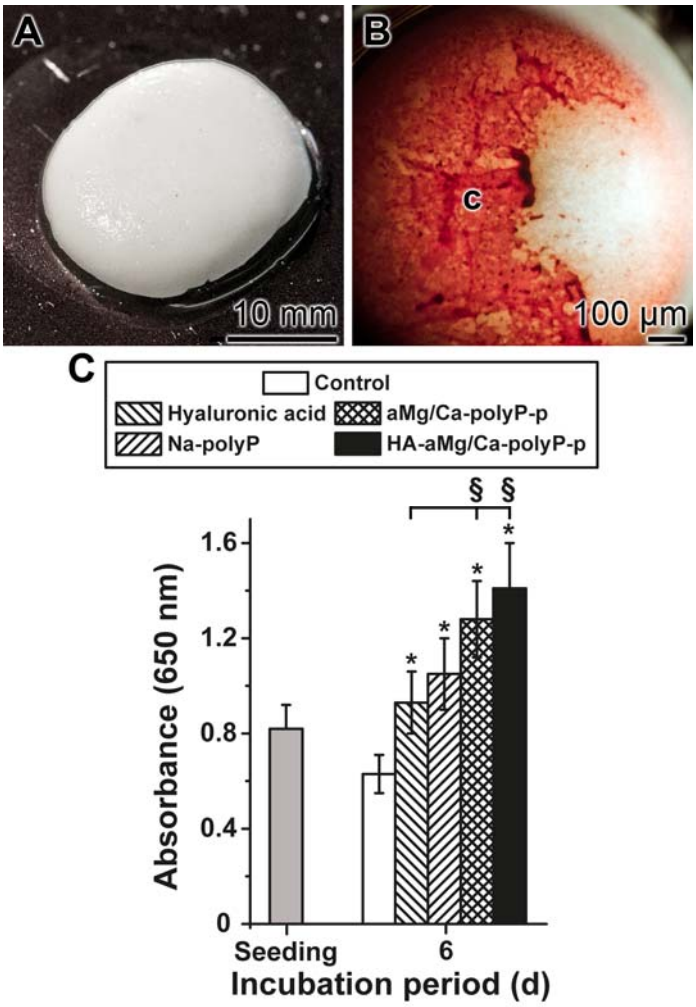

Fig. 10. Steady-state expression levels of the (**A**) *ALP* gene, (**B**) *collagen type II alpha 1* (*COL2A1*) gene, (**C**) *collagen type III alpha 1* (*COL3A1*) gene as well as the (**D**) *aggrecan gene* in chondrocytes. The cells were incubated for 12 d or 21 d in the absence of the components (open bars), or the presence of 0.5 mg/ml hyaluronic acid (hatched to the right), 50 µg/ml of Na-polyP (hatched to the left), of aMg/Ca-polyP-p (cross-hatched) or of HA-aMg/Ca-polyP-p (filled). After the incubation the cells were harvested, their RNA extracted and subjected to qRT-PCR analyses. The expression values are given as ratios to the reference gene GAPDH; the ratios at time zero are in grey. The results are means from 5 parallel experiments; * $P < 0.01$; the values are computed against the expression measured in cells during seeding.

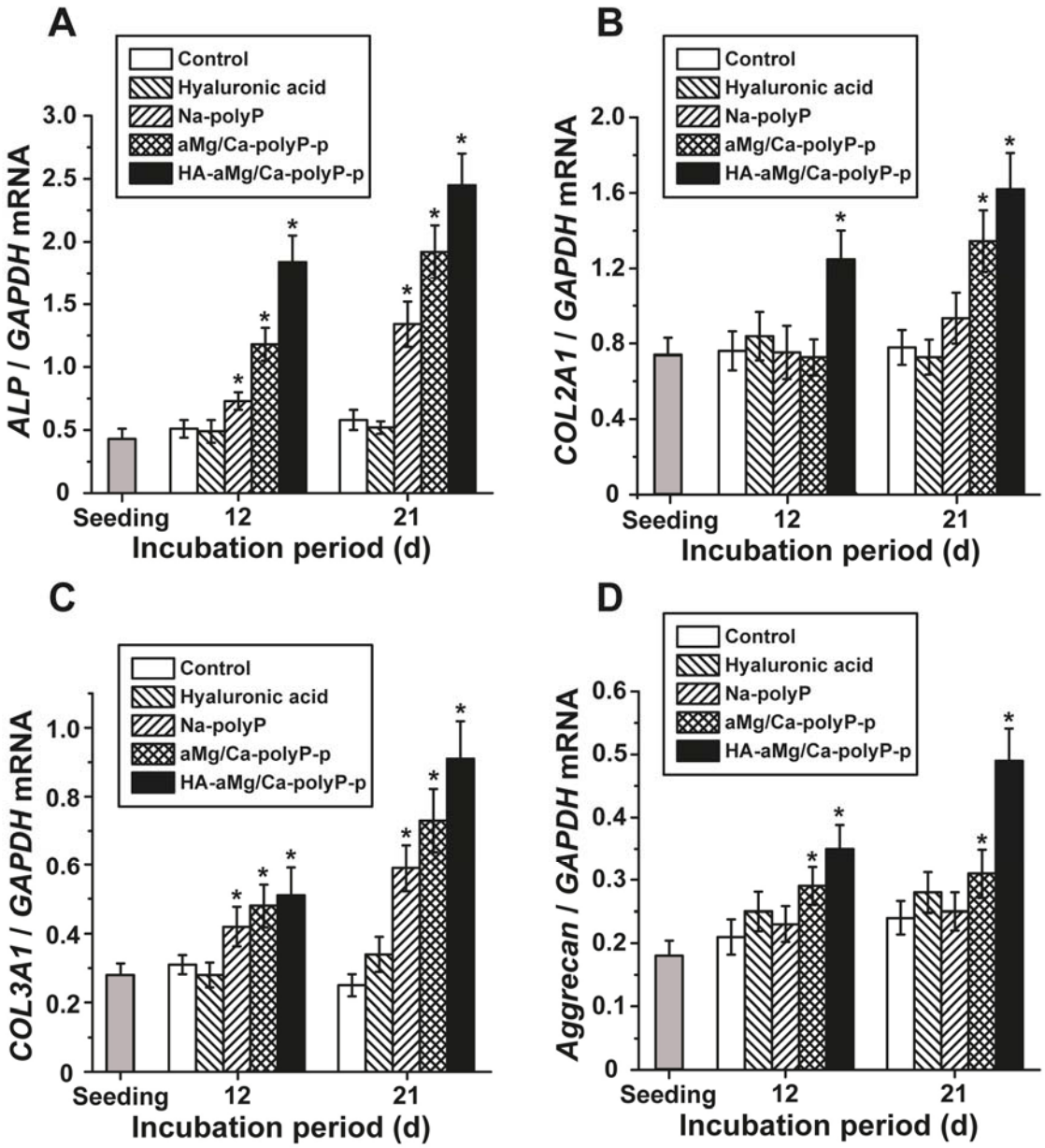

Fig. 11. Schematic outline of the proposed changes of amorphous Mg-polyP microparticles (aMg-polyP MP) undergoing in the synovial fluid. It is depicted that the microparticles formed of Mg-polyP readily dissolve partially and allow the Ca^{2+} ions as well as the hyaluronic acid polyanion, both present in the synovial fluid to bind to the polyanion polyP. After the exchange of Mg^{2+} by Ca^{2+} and inclusion of the hyaluronic acid polymers into the polyP core HA-aCa-polyP microparticles are formed; they surely contain still remaining Mg^{2+}.