Title of the Invention: Printable morphogenetic phase-specific chitosan-calcium-polyphosphate scaffold for bone repair
Abstract Title: Preparation of a tissue scaffold by 3D printing

This invention concerns a formula for the synthesis of a printable hybrid material, formed of carboxymethyl chitosan (CMC) and polyphosphate (polyP). Both polymers are linked together by calcium ions. The inventive CMC-polyP material, in combination with alginate, is biocompatible, biodegradable and useful for three-dimensional (3D) printing and 3D cell printing (bioprinting). The CMC-polyP scaffold, hardened by exposure to calcium ions, is morphogenetically active and can be used in bone tissue engineering, as a biomimetic 3-phase scaffold that mimics and induces essential phases in bone repair, including blood clot formation and platelet degranulation (release of growth factors and cytokines) (Phase 1: initiation phase), calcium carbonate bioseed formation (Phase 2: nucleation) and expression / activation of bone alkaline phosphatase (Phase 3: hydroxypatite – biomineral formation).
Figure 3

A

B

C

D
Figure 4

[Bar chart showing AR (nmoles/µg DNA) over Incubation period (d) for different treatments: control (- OC), + OC; N,O-CMC hg, + OC; N,O-CMC layer - polyP, + OC; N,O-CMC layer + polyP. The chart includes error bars and asterisks to indicate significant differences.]
Figure 5

Hemoglobin absorbance (OD$_{540}$)

- Chitosan control
- N,O-CMC hg
- N,O-CMC layer - polyP
- Chitosan + polyP
- N,O-CMC layer + polyP

* indicates significant difference compared to control.
PRINTABLE MORPHOGENETIC PHASE-SPECIFIC CHITOSAN-CALCIUM-POLYPHOSPHATE SCAFFOLD FOR BONE REPAIR

This invention concerns a formula for the synthesis of a printable hybrid material, formed of carboxymethyl chitosan (CMC) and polyphosphate (polyP). Both polymers are linked together by calcium ions. The inventive CMC-polyP material [composition of CMC with polyP], in combination with alginate, is biocompatible, biodegradable and useful for three-dimensional (3D) printing and 3D cell printing (bioprinting). The CMC-polyP scaffold, hardened by exposure to calcium ions, is morphogenetically active and can be used in bone tissue engineering, as a biomimetic 3-phase scaffold that mimics and induces essential phases in bone repair, including blood clot formation and platelet degranulation (release of growth factors and cytokines) (Phase 1: initiation phase), calcium carbonate bioseed formation (Phase 2: nucleation) and expression / activation of bone alkaline phosphatase (Phase 3: hydroxyapatite – biomineral formation).

**Background of Invention**

Biological bone substitutes must meet the requirements to be highly porous and to offer a microenvironment for regenerative cells, e.g. support cell attachment, proliferation, differentiation, and, by that, initiate and maintain neo-tissue genesis. To fabricate a three-dimensional (3D) scaffold for tissue engineering various metals have been exploited. In spite of their advantageous mechanical properties metals have the disadvantage not to be biodegradable. In parallel, inorganic/ceramic materials, e.g. hydroxyapatite (HA) or calcium phosphates, have been developed that display the desired osteoconductivity, but are difficult to produce in a highly porous structure and are brittle. Finally, biomimetic artificially designed scaffolds that mimic the structures of living systems provide the feature of the physiological extracellular matrix to recruit cells in the implanted biomaterial. In this context, chitosan derived from chitin, is of particular interest.

**Chitosan**

Chitosan is a polysaccharide derived from chitin that is randomly built by β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units. Chitosan shows suitable properties for tissue engineering purposes. This polymer is biocompatible and biodegradable, and can be used both for 3D-scaffolds, as gels and tissue-like units, and for 2D-scaffolds, as films and fibers (Crisier F, Jérôme C. Chitosan-based biomaterials for tissue engineering. Europ Polymer J 2013;49:780-792). Chitosan has been used for space-filling implants. However, this natural polymer has to be processed with morphogenetically active components, e.g. silica, to become a suitable matrix for bone regeneration (Shirosaki Y, Tsuru K, Hayakawa S, Osaka A, Lopes MA, Santos JD, Costa MA, Fernandes MH. Physical, chemical and in vitro biological profile of chitosan hybrid membrane as a function of organosiloxane concentration. Acta Biomater 2009; 5:346-355).

**Essential phases during bone repair**

Bone repair is a process that can be divided in multiple phases that could be affected by “intelligent”, phase-specific scaffold materials.

**Phase 1 - Blood coagulation and effect of polyP**: Bone repair is initiated by blood coagulation at the site of the bone defect. The dense granules of human platelets contain
substantial amounts of polyP, with chain lengths of 70-75 (Ruiz FA, Lea CR, Oldfield E, Docampo R. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 2004;279:44250-44257) or 60-100 phosphate units (Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, Schmidbauer S, Gahl WA, Morrissey JH, Renné T. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 2009;139:1143-1156), which is released upon platelet activation (Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci USA 2006;103:903-908). PolyP secreted by platelets acts as a hemostatic regulator; it is a procoagulant agent that accelerates blood clotting by promoting the activation of factor V and activation of the contact pathway (Smith SA, Morrissey JH. Polyphosphate as a general procoagulant agent. J Thromb Haemost 2008;6:1750-1756). On the other hand, polyP delays clot lysis by enhancing the thrombin-activatable fibrinolysis inhibitor (Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci USA 2006;103:903-908). A series of growth factors are found in blood clot, including fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), transforming growth factor beta (TGF-b) and vascular endothelial growth factors (VEGF). These factors can enhance different phases of osteogenesis. For example, the proliferation of osteoblastic progenitor cells is stimulated by PDGF, EGF and FGF-2.

**Phase 2 – Calcium carbonate bioseed formation:** It could be demonstrated that biocalcrite (CaCO$_3$) fulfills crucial roles during initiation of bone HA formation. It has been demonstrated that CaCO$_3$ deposits function as bio-seeds for Ca-phosphate precipitation onto bone forming cells (Müller WEG, Schröder HC, Schloessmacher U, Grebenjuk VA, Ushijima H, Wang XH. Induction of carbonic anhydrase in SaOS-2 cells, exposed to bicarbonate and consequences for calcium phosphate crystal formation. Biomaterials 2013;34:8671-8680). In particular, the carbonic anhydrase not only facilitates bicarbonate/calcium carbonate biomineral formation but also acts in concert with the polyP / pyrophosphate-degrading bone alkaline phosphatase (tissue-nonspecific ALP), through the initial formation of Ca-carbonate deposits.

**Phase 3 – Hydroxyapatite deposition:** The initially formed Ca-carbonate deposits are subsequently transformed into Ca-phosphate/HA minerals by the ALP, opening the development of new strategies for therapeutic intervention of bone diseases, such as the development of morphogenetically active implant materials (Wang XH, Schröder HC, Müller WEG. Enzyme-based biosilica and biocalcite: biomaterials for the future in regenerative medicine. Trends Biotechnol 2014, in press; doi: 10.1016/j.tibtech.2014.05.004).

**Polyphosphate (polyP)**

Previously the inventors published the role of polyP during HA deposition on bone cells. PolyP is a linear polymer occurring in nature of two up to hundreds of phosphate residues (Schröder HC, Müller WEG, eds. Inorganic Polyphosphates - Biochemistry, Biology, Biotechnology. Prog Mol Subcell Biol 1999;23:45-81). PolyP can be synthesized both chemically and enzymatically (Kulaev IS, Vagabov V, Kulakovskaya T. The Biochemistry of Inorganic Polyphosphates. New York: John Wiley & Sons Inc; 2004).

Several enzymes that degrade polyP are known (e.g., Lorenz B, Müller WEG, Kulaev IS, Schröder HC. Purification and characterization of an exopolyphosphatase activity from Saccharomyces cerevisiae. J Biol Chem 1994;269:22198-22204), among them the bone ALP (Lorenz B, Schröder HC. Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase. Biochim Biophys Acta 2001;1547:254-261).

The bone ALP (tissue-nonspecific ALP) is an exopolyphosphatase that degrades polyP by a processive mechanism to monomeric phosphate (Lorenz B, Schröder HC. Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase. Biochim Biophys Acta 2001;1547:254-261).


PolyP is morphogenetically active after complex formation with Ca^{2+} ions (polyP•Ca^{2+}-complex or polyP•Ca^{2+}-salt); the polyP•Ca^{2+}-complex

- induces the expression and enhances the activity of the bone ALP (tissue non-specific ALP) (Müller WEG, Wang XH, Diehl-Seifert B, Kropf K, Schloßmacher U, Lieberwirth I, Glasser G, Wiens M, Schröder HC. Inorganic polymeric phosphate/polyphosphate is an inducer of alkaline phosphatase and a modulator of intracellular Ca^{2+} level in osteoblasts (SaOS-2 cells) in vitro. Acta Biomater 2011;7:2661-2671);
- increases the intracellular Ca^{2+} level in osteoblasts (Müller WEG, Wang XH, Diehl-Seifert B, Kropf K, Schloßmacher U, Lieberwirth I, Glasser G, Wiens M, Schröder HC. Inorganic polymeric phosphate/polyphosphate is an inducer of alkaline phosphatase and a modulator of intracellular Ca^{2+} level in osteoblasts (SaOS-2 cells) in vitro. Acta Biomater 2011;7:2661-2671);

The following patent applications concerning polyP or polyP•Ca^{2+}-complex are relevant:

GB1406840.7. Morphogenetically active hydrogel for bioprinting of bioartificial tissue. Inventors: Müller WEG, Schröder HC, Wang XH.

GB1319416.2. Modulator of bone mineralization based on a combination of polyphosphate/carbonate and carbonic anhydrase activators. Inventors: Müller WEG, Schröder HC, Wang XH.

In this invention, a complex of polyP with chitosan is described that can be used as a biomimetic material for bone tissue engineering and repair that features controlled morphology and displays morphogenetic activity.


Biosilica


Biosilica has an inductive anabolic effect on bone-forming cells; it increases the expression of BMP-2 and causes a shift of the osteoprotegerin : RANKL ratio, resulting in an inhibition of differentiation of pre-osteoclasts into mature osteoclasts (reviewed in: Wang XH, Schröder HC, Wiens M, Ushijima H, Müller WEG. Bio-silica and bio-polyphosphate: applications in biomedicine (bone formation). Curr Opin Biotechnol 2012;23:570-578).

The following patents or patent applications concerning biosilica are relevant:


DE10246186. In vitro and in vivo degradation or synthesis of silicon dioxide and silicones, useful e.g. for treating silicosis or to prepare prosthetic materials, using a new silicate enzyme. Inventors: Müller WEG, Kraska A, Schröder HC.


EP09005849.6. Use of silintaphin for the structure-directed fabrication of (nano)composite materials in medicine and (nano)technology. Inventors: Wiens M, Müller WEG, Schröder HC, Wang X.
Biogllass


The following patent application concerning bioglasses is relevant:

GB1408402.4. 3D cell printing of bioglass-containing scaffolds by combination with cell-containing morphogenically active alginate/gelatin hydrogels. Inventors: Müller WEG, Schröder HC, Wang XH.

Biomimetic materials gain increasing importance in tissue engineering since they may represent regenerative alternatives to harvested tissues for transplantation. Among the three-dimensional templates, mimicking the physiological extracellular matrix, chitosan and \(N,\text{O-carboxymethyl chitosan (N,O-CMC)}\) are widely used. In order to provide these polymers with a biological function additional components have to be added.

The inventors developed a formula for the preparation of a bioprintable material, composed of alginate, \(N,\text{O-CMC}\) and Na-polyP. After printing of this material to custom-designed/fabricated layers and implants, the structures are exposed to \(\text{Ca}^{2+}\) in order to harden them. During \(\text{Ca}^{2+}\) exposure the \(\text{Na}^+\) cations in the polyP are exchanged by \(\text{Ca}^{2+}\) allowing the bridging of polyP to \(N,\text{O-CMC}\) and rendering the composite material particularly stable without loosing the biological activity of polyP.

The inventors describe the formulation and fabrication of \(N,\text{O-CMC-based polyP hybrid material. The two polymers are linked together via Ca}^{2+}\) bridges in a stable way and provide a porous structure. The material can be printed to implants filling μCT analyzed lesions. Since the material retains its biological morphogenetic function, initiating biomineralization onto SaOS-2 bone-like cells, and accelerates blood clotting, \(N,\text{O-CMC-polyP}\) represents a promising new material applicable in tissue engineering of bone defects.

**Detailed description of the invention**

This invention is related to the formula for the synthesis of a new hybrid material, formed of \(N,\text{O-CMC}\) and polyphosphate (polyP), a natural polymer. Both polymers are linked together via \(\text{Ca}^{2+}\) bridges. Those \(N,\text{O-CMC-polyP}\) materials retain their morphology in culture medium and are especially useful for bioprinting. The \(N,\text{O-CMC-polyP}\) printed layers and tissue units also retain their biological function, to induce bone cells to biomineralization, and to
accelerate the clotting process of human blood and, in turn, represent a promising new material useful for tissue engineering purposes.

The inventive scaffold consists of carboxymethyl chitosan, polyphosphate (sodium salt), and alginate (sodium salt), and is fabricated by 3D printing (bioprinting) of the resulting hydrogel and subsequent hardening by exposure to calcium ions.

The carboxymethyl chitosan can be formed by carboxymethylation of the amino groups of chitosan (N-carboxymethyl chitosan) or the hydroxy groups of chitosan (O-carboxymethyl chitosan) or both (N,O-carboxymethyl chitosan).

The non-carboxymethylated amino groups and / or the non-carboxymethylated hydroxy groups of the carboxymethyl chitosan can be acetylated or partially acetylated.

The novel biomimetic 3-phase scaffold according to this invention has the following properties; it is:

- biocompatible,
- biodegradable, and
- printable.

This scaffold mimics three essential phases in bone repair; it affects the following 3 target sites which are active during 3 phases of bone repair:

**Phase 1:** Clot formation associated with the release of growth factors/cytokines from platelets (initiation phase)

**Phase 2:** Calcium carbonate bioseed formation by providing nucleation centers at the carboxymethyl chitosan backbone (seed phase)

**Phase 3:** Expression / activation of bone alkaline phosphatase (Hydroxyapatite – biomineral formation phase)

The finding that the inventive material, containing polyP linked via Ca$^{2+}$ to the N,O-CMC polymer, has a significantly stronger effect than the published chitosan+polyP-PEC complex (Mi FL, Shyu SS, Wong TB, Jang SF, Lee ST, Lu KT. Chitosan-polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using tripolyphosphate or polyphosphate as reagent. J Appl Polymer Sci 1999;74:1093-1107; Ong SY, Wu J, Moochhala SM, Tan MH, Lu J. Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. Biomaterials 2008;29:4323-4332) was unexpected, for the following reasons: (i) From published results, it is likely that the effect of polyP on clot formation does not require complex or salt formation of the polyP with calcium ions; in the contrary, the effect of polyP on coagulation has even been observed after preincubation of plasma before addition of calcium ions (Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci USA 2006;103:903-908). (ii) Moreover, it is surprising that polyP, in the form of a calcium salt, is biologically active even after complex formation with the N,O-CMC polymer.
In addition, it was surprising that the inventive formulation (i) is printable and (ii) the printed meshwork shows sufficient stability after 3D printing – in contrast to the predictions based on the properties of the individual materials alone.

The alginate can be supplemented with gelatin or another collagen-derived product.

In addition, the inventors show that the inventive alginate-CMC-polyP hydrogel can be supplemented with silica or biosilica that stimulates bone-forming cells to mineralize and to express morphogenetically active cytokines, e.g. BMP-2. The polymeric silica or biosilica can be enzymatically formed by silicatein.

The technology according to this invention can be applied for the fabrication of cell-containing scaffold/implants, in particular scaffolds containing bone-forming cells or bone-dissolving cells or a mixture of these cells, whereby the cells are suspended in the alginate hydrogel and the resulting cell-containing alginate-CMC-polyP hydrogel is subjected to 3D printing (bioprinting) and subsequent hardening by exposure to calcium ions.

The hydrogel can be simultaneously printed, using a 3D printing technique, with a suspension of bioglass (bioactive glass) (nano)particles that can be composed of SiO₂:CaO:P₂O₅ or SiO₂:Na₂O:CaO:P₂O₅ of various molar ratios, for example SiO₂:CaO:P₂O₅ of a molar ratio (mol.%) of 55:40:5 or SiO₂:Na₂O:CaO:P₂O₅ of a molar ratio (mol.%) of 46.1:24.4:26.9:2.6 (45S5 Bioglass®).

The average chain lengths of the polyP molecules can be in the range 10 to up to 100 phosphate units. Optimal results were obtained with polyP molecules with an average chain length of about 40 phosphate units.

The polymeric silicic acid that can be added as an additional component can be formed by an enzyme or protein involved in biosilica (amorphous, hydrated silicon oxide) metabolism, such as silicatein or a silicatein fusion protein. The silicatein polypeptide or a silicatein fusion protein can be produced using a prokaryotic or eukaryotic expression system, or can be produced synthetically.

The silicatein or silicatein fusion protein can be present together with a suitable substrate (silica precursor) such as water glass, orthosilicic acid, orthosilicates, monoalkoxyxilanoltriols, dialkoxyxilanoltriols, trialkoxyxilanol, tetraalkoxyxilanol, alkyl-silanetriols, alkyl-silanediols, alkyl-monoalkoxyxilanoltriols, alkyl-monoalkoxyxilanol, alkyl-dialkoxyxilanol, or alkyl-trialkoxyxilanol.

A further aspect of the invention concerns a 3D-bioprinted scaffold obtained by one of the methods described above, used as a bone implant or a material forming part of such implant.

The bone implant material can be produced for the treatment of a bone defect in the form of a customized implant by 3D printing, 3D cell printing (bioprinting) or another rapid prototyping procedure.

The invention will now be described further in the following preferred examples, nevertheless, without being limited thereto. For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties. In the Figures,
**Figure 1** shows the formation of $N,O$-CMC-polyP membranes and tissue units. (A) Chitosan, characterized by the D-glucosamine (deacetylated) and $N$-acetyl-D-glucosamine (acylated) units, is converted into $N,O$-CMC by partial carboxymethylation of the polymer. (B to E) Mats of two (B and C) to six layers (D) were bioprinted. (E) Printing of a $N,O$-CMC-polyP tissue-like unit, an implant (im), formed according to the lesion in a pig underjaw (uj).

**Figure 2** shows the EDX analysis of membranes formed of $N,O$-CMC. The membranes were prepared in the absence of polyP (A and C) or in the presence of polyP (B and D), only in the EDX spectrum of the $N,O$-CMC-polyP membranes the signals for phosphorous and calcium show up.

**Figure 3** shows the integrity and stability of the $N,O$-CMC-polyP meshwork. In contrast to the scaffold meshes build from (A) $N,O$-CMC, not containing polyP, which fuse, the $N,O$-CMC-polyP meshes remain intact even if submerged in culture medium. (B and C) Freshly prepared $N,O$-CMC-polyP meshwork. It is seen that only at the crossing points a fusion of the printed cylinders is seen; a continuous crossing point is formed. (D) Even after an incubation period of the $N,O$-CMC-polyP mesh in culture medium for 5 d, the cylinders remains separated and allow the cells (c) to proliferate in the open space.

**Figure 4** shows the potency of SaOS-2 cells to mineralize on chitosan matrices. The SaOS-2 cells were grown in the absence (control; -OC) or presence of the OC. In the latter assays the cells were cultured on the previously published $N,O$-CMC hydrogel ($N,O$-CMC hg), or the $N,O$-CMC layers, in the absence ($N,O$-CMC - polyP) or presence of polyP ($N,O$-CMC + polyP). The extent of biomineralization (Alizarin Red S [AR]) is correlated with the DNA content in the assays. Values represent the means ($\pm$SD) from 10 separate experiments each. The $N,O$-CMC-polyP matrix significantly increases the mineralization; *$P < 0.01$.

**Figure 5** shows the effect of chitosan polyP complex ("Chitosan+polyP”), $N,O$-CMC hydrogel ("$N,O$-CMC hg") and $N,O$-CMC layers minus polyP ("$N,O$-CMC layer - polyP") and plus polyP ("$N,O$-CMC layer + polyP") on blood clotting rates. In the control non-processed chitosan had been added. The absorbance of hemoglobin from lysed noncoagulated erythrocytes was determined. The significance was compared to the chitosan control; *$p < 0.05$.

**Examples**

In the following examples, only the inventive method described, using $N,O$-CMC-polyP membranes, layers and tissue-like-blocks. Nevertheless, the method according to this invention can also be applied using O-carboxymethyl chitosan (O-CMC) and N-carboxymethyl chitosan (N-CMC) and polyP molecules with chain lengths other than 40 phosphate units, and the person of skill will be able to adjust the method as described accordingly.

**Preparation of $N,O$-CMC-polypophosphate membranes, layers and tissue-like-blocks**

As described under “Methods” the $N,O$-CMC-polyP was prepared. The $N,O$-CMC was mixed with Na-polyP; then the two polymers were linked together via Ca$^{2+}$ ionic bridges. The membranes, layers or tissue-like-blocks were analyzed for the presence of phosphorus by EDX spectroscopy. As an example the EDX spectra from membranes, prepared without NapolyP and with Na-polyP are given (Figure 2A and B). The surfaces of the membranes were analyzed. The spectra show that the membranes that had been formed in the presence of NapolyP, and then linked via Ca$^{2+}$ to the $N,O$-CMC polymer showed the signals for phosphorous
and calcium (Figure 2D), while those signals are absent in the membranes formed in the absence of polyP (Figure 2C).

**Printing of N,O-CMC-polyphosphate layers and tissue-like-blocks**

Using the setting as described above, two or six layers were printed and used for the *in vitro* assays. In Figure 1B and C the two-layer mats for the *in vitro* studies are shown. The mesh size of the cylinders was =0.5 x 0.5 mm.

The thickness of the layers can be increased by increasing the numbers of layers. A six-layer pad is shown in Figure 2D. Increasing further the layering, tissue-like blocks are formed (Figure 1E). Here the inventors printed a cranial defect in a pig underjaw, after having analyzed the lesion by μCT.

**N,O-CMC-polyP layers assayed in cell culture**

The two-layer printed scaffolds were used for the cell culture experiments. If a sample from a N,O-CMC layer, lacking any polyP, has been printed the cylinders fuse in the culture medium (Figure 3A). In contrast, if this material to be printed is supplemented with polyP, the N,O-CMC-polyP, then the cylinders remain separated (Figure 1B and D). Even more, the crossing cylinders fuse only at the intimate, initial crossing points, under formation of continuous attachment mesh between the two layers (Figure 3B and C). The distinct intersections between the printed cylinders leave room for the infiltration of cells (Figure 3D). Even after a five days’ incubation period the meshwork remain intact (Figure 3D).

**Mechanical properties of the N,O-CMC-polyP material**

The hardness of the N,O-CMC-polyP scaffold was measured with an indenter device and using a cantilever on the top of a glass ferrule. Scaffold samples of 6 layers with a thickness of 2 mm were measured. The samples that had been obtained immediately after printing were submerged into saline and tested for the hardness, using the reduced Young's modulus [RedYM] as a parameter. If those samples, N,O-CMC-polyP were analyzed a mechanical RedYM stiffness 935±128 kPa was determined (n=10); in contrast, the samples from the scaffold lacking the polyP measured only 27±3 kPa. In comparison, and using the same settings the trabecular bone from a rabbit tissue was found to have a modulus of 2,300 kPa. Submersing the N,O-CMC-polyP scaffold samples in simulated body fluid (Kokubo T. Bioactive glass ceramics: properties and applications. Biomaterials 1991,12:155-163) the RedYM stiffness changed not significantly during a 3 weeks’ period; the values are around 900±115 kPa; only after 6 weeks a significant reduction to 686±102 kPa is measured.

**Mineralization of SaOS-2 cells on N,O-CMC matrices**

The matrices as prepared here, N,O-CMC without and with polyP, as well as (in comparison) the chitosan preparation, termed N,O-CMC hydrogel, published earlier (Chen XG, Park HJ. Chemical characteristics of O-carboxymethyl chitosans related to the preparation conditions. Carbohydrate Polymers 2003;53:355-359, Luo Y, Teng Z, Wang X, Wang Q. Development of carboxymethyl chitosan hydrogel beads in alcohol-aqueous binary solvent for nutrient delivery applications. Food Hydrocolloids 2013a;31: 332-339), were tested for their potency to induce in SaOS-2 cells biomineralization. The cells were transferred after an initial incubation period for 3 d in medium/FCS supplemented with the OC.

As shown in Figure 4, N,O-CMC-polyP, which contains polymeric polyP bound to the N,O-CMC, caused a significantly higher induction of the mineralization of the cells (0.93±0.09 nmols of Alizarin Red S bound to the cells [based on μg of DNA] at day 8) than N,O-CMC matrices, lacking polyP. This holds true for the published N,O-CMC hydrogel matrix.
(0.38±0.04 nmoles/µg), and the matrix prepared here (0.46±0.06 nmoles/µg). In the absence of any chitosan matrix the extent of mineralization was 0.38±0.07 nmoles/µg (not shown in Figure 4). In the absence of the OC the level of mineralization was low with =0.20±0.03 nmoles/µg.

Effect of N,O-CMC-polyP on kinetics of blood clotting

PolyP is known to promote clot formation (Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci USA 2006;103:903-908) and also to reverse anticoagulation and bleeding episodes in patients with hemophilia (Smith SA, Morrissey JH. Polyphosphate as a general procoagulant agent. J Thromb Haemost 2008;6:1750-1756). In turn the inventors measured here the effect of the different matrices on the clotting time of human blood in vitro. The determinations were performed with whole blood contacted with similar amounts of matrices. After a contact period of 10 min the remaining erythrocytes in suspension were measured on the basis of their hemoglobin content (Figure 5). It is obvious that those samples that contain no polyP (“Chitosan control”, “N,O-CMC hydrogel” [Chen XG, Park HJ. Chemical characteristics of O-carboxymethyl chitosans related to the preparation conditions. Carbohydrate Polymers 2003;53:355-359; Luo Y, Teng Z, Wang X, Wang Q. Development of carboxymethyl chitosan hydrogel beads in alcohol-aqueous binary solvent for nutrient delivery applications. Food Hydrocolloids 2013a;31: 332-339] and N,O-CMC layers, prepared here minus polyP “N,O-CMC layer - polyP”) did not change significantly the hemoglobin concentration, as a measure for the free erythrocyte number. However, the N,O-CMC layers, prepared in the present contribution plus polyP “N,O-CMC layer + polyP” and the chitosan polyelectrolyte complex (PEC) containing polyP “Chitosan+polyP” significantly reduced the number of free erythrocytes, and conversely increased the number of erythrocytes bound to the matrices. Comparing the two samples, polyP linked via Ca\(^{2+}\) to the N,O-CMC polymer significantly reduced the number of free erythrocytes (0.43±0.07 versus 0.85±0.12), compared to the Chitosan+polyP-PEC sample (Mi FL, Shyu SS, Wong TB, Jang SF, Lee ST, Lu KT. Chitosan-polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using tripolyphosphate or polyphosphate as reagent. J Appl Polymer Sci 1999;74:1093-1107; Ong SY, Wu J, Moochhala SM, Tan MH, Lu J. Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. Biomaterials 2008;29:4323-4332).

The finding of the inventors that polyP linked via Ca\(^{2+}\) to the N,O-CMC polymer causes a significantly higher reduction of the erythrocyte number (increase in clotting time) than the published chitosan+polyP-PEC complex (Figure 5) was unexpected, in particular because it has not been described that the effect of polyP on blood coagulation requires a complex or salt formation of the polyP with calcium ions. The effect of polyP on the clotting time seems to be mediated by a calcium-independent mechanisms (Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH (2006) Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci USA 103:903-908). In addition, it was surprising that polyP, as a calcium complex/salt, is biologically active even after binding to the N,O-CMC polymer.

The following example shows that the inventive method described can also be applied, if polyP linked via Ca\(^{2+}\) to the N-carboxymethylated chitosan (N-CMC-polyP) or polyP linked via Ca\(^{2+}\) to the O-carboxymethylated chitosan (O-CMC-polyP) instead of N,O-CMC-polyP is used.
The effects of \(N\)-CMC-polyP and \(O\)-CMC-polyP on mineralization of SaOS-2 cells are summarized in Table 1. Both preparations show a significantly higher effect on mineralization than the published chitosan polyP complex (Mi FL, Shyu SS, Wong TB, Jang SF, Lee ST, Lu KT. Chitosan-polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using tripolyphosphate or polyphosphate as reagent. J Appl Polymer Sci 1999;74:1093-1107). However, they were less efficient than \(N\)-CMC-polyP.

These data surprisingly show that polyP, linked to \(N\)-CMC or \(O\)-CMC via \(\text{Ca}^{2+}\)-linkages has a significantly higher biomineralization potential than when linked otherwise.

Table 1. Effect of chitosan polyP complex (“Chitosan+polyP”), and \(N\)-CMC layers minus polyP (“\(N\)-\(N\)-CMC layer - polyP”), \(N\)-CMC layers minus polyP (“\(N\)-\(N\)-CMC layer - polyP”), \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer - polyP”), and \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer - polyP”), \(N\)-CMC layers minus polyP (“\(N\)-\(O\)-CMC layer + polyP”), \(N\)-CMC layers minus polyP (“\(N\)-\(O\)-CMC layer + polyP”), \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer + polyP”), and \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer + polyP”) on mineralization of SaOS-2 cells. The cells were grown in the absence (control) or presence of the OC (other assays). The extent of biomineralization (Alizarin Red S [AR]) is correlated with the DNA content. The incubation period was 8 days.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AR (nmoles/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>Chitosan+polyP</td>
<td>0.38±0.04</td>
</tr>
<tr>
<td>(N)-(O)-CMC layer - polyP</td>
<td>0.46±0.06</td>
</tr>
<tr>
<td>(N)-(N)-CMC layer - polyP</td>
<td>0.42±0.10</td>
</tr>
<tr>
<td>(O)-(O)-CMC layer - polyP</td>
<td>0.40±0.09</td>
</tr>
<tr>
<td>(N)-(O)-CMC layer + polyP</td>
<td>0.93±0.09</td>
</tr>
<tr>
<td>(N)-(O)-CMC layer + polyP</td>
<td>0.80±0.11</td>
</tr>
<tr>
<td>(O)-(O)-CMC layer + polyP</td>
<td>0.77±0.10</td>
</tr>
</tbody>
</table>

The effects of \(N\)-CMC-polyP and of \(O\)-CMC-polyP on the kinetics of blood clotting are summarized in Table 2. Both preparations, like \(N\)-\(O\)-CMC-polyP, cause a significantly higher effect on blood clotting rates than the published chitosan polyP complex (Mi FL, Shyu SS, Wong TB, Jang SF, Lee ST, Lu KT. Chitosan-polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using tripolyphosphate or polyphosphate as reagent. J Appl Polymer Sci 1999;74:1093-1107), but they are less effective compared to \(N\)-\(O\)-CMC-polyP.

Table 2. Effect of chitosan polyP complex (“Chitosan+polyP”), and \(N\)-CMC hydrogel (“\(N\)-\(N\)-CMC hg”), \(N\)-CMC hydrogel (“\(N\)-\(N\)-CMC hg”), \(O\)-CMC hydrogel (“\(O\)-\(O\)-CMC hg”), and \(N\)-CMC layers minus polyP (“\(N\)-\(O\)-CMC layer - polyP”), \(N\)-CMC layers minus polyP (“\(N\)-\(O\)-CMC layer - polyP”), \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer - polyP”), \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer - polyP”), \(N\)-CMC layers minus polyP (“\(N\)-\(O\)-CMC layer + polyP”), \(N\)-CMC layers minus polyP (“\(N\)-\(O\)-CMC layer + polyP”), \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer + polyP”), \(O\)-CMC layers minus polyP (“\(O\)-\(O\)-CMC layer + polyP”) on blood clotting rates. In the control non-processed chitosan had been added. The absorbance of hemoglobin from lysed noncoagulated erythrocytes was determined.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hemoglobin absorbance (OD_{540})</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.42±0.13</td>
</tr>
<tr>
<td>Chitosan+polyP</td>
<td>0.85±0.12</td>
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<tr>
<td></td>
<td>Value</td>
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<tr>
<td>----------</td>
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<tr>
<td>N,O-CMC hg</td>
<td>1.29±0.15</td>
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<tr>
<td>N-CMC hg</td>
<td>1.40±0.21</td>
</tr>
<tr>
<td>O-CMC hg</td>
<td>1.33±0.17</td>
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<tr>
<td>N,O-CMC layer - polyP</td>
<td>1.47±0.17</td>
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<tr>
<td>N-CMC layer - polyP</td>
<td>1.51±0.20</td>
</tr>
<tr>
<td>O-CMC layer - polyP</td>
<td>1.41±0.20</td>
</tr>
<tr>
<td>N,O-CMC layer + polyP</td>
<td>0.43±0.07</td>
</tr>
<tr>
<td>N-CMC layer + polyP</td>
<td>0.61±0.11</td>
</tr>
<tr>
<td>O-CMC layer + polyP</td>
<td>0.58±0.08</td>
</tr>
</tbody>
</table>

**Methods**

**Polyphosphate**

The sodium polyphosphate (Na-polyP of an average chain of 40 phosphate units) used in the Examples has been obtained from Chemische Fabrik Budenheim (Budenheim; Germany).

**Preparation of N,O-carboxymethyl chitosan**

N,O-carboxymethyl chitosan (N,O-CMC) can be prepared from chitosan according to state-of-the-art procedures (Chen XG, Park HJ). Chemical characteristics of O-carboxymethyl chitosans related to the preparation conditions. Carbohydrate Polymers 2003;53:355-359; Chen SC, Wu YC, Mi FL, Lin YH, Yu LC, Sung HW. A novel pH-sensitive hydrogel composed of N,O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. J Control Release 2004;96:285-300; Sakairi N, Suzuki S, Ueno K, Han SM, Nishi N, Tokura S. Biosynthesis of hetero-polysaccharides by *Acetobacter xylinum*-synthesis and characterization of metal-ion adsorptive properties of partially carboxymethylated cellulose. Carbohydrate Polymers 1998;37:409-414). In brief, 10 g of chitosan (e.g. from shrimp shells, ≥75% (deacetylated) [deacetylated chitin]) is added to water/isopropanol mixture (20 ml: 40 ml) containing 14.1 g NaOH and kept at room temperature for 1 h under mild stirring. After that, 15 g of monochloroacetic acid dissolved in 20 ml of isopropanol is added to the mixture dropwise. The reaction mixture is heated to 50°C and stirring is continued for 4 h. Then the materials is filtered and washed three times with 80% ethyl alcohol. The resulting solid is dried overnight in an oven at 60°C to obtain the Na salt of N,O-CMC. For the conversion to the H-form of N,O-CMC, the obtained powder is suspended in 100 ml of aqueous 80% ethyl alcohol solution. Then 10 ml hydrochloric acid (37%) is added and stirred for 30 min. Finally, the suspension is filtrated and washed with ethyl alcohol until a neutral pH is obtained; the material is dried at 60°C overnight (Sakairi N, Suzuki S, Ueno K, Han SM, Nishi N, Tokura S. Biosynthesis of hetero-polysaccharides by *Acetobacter xylinum*-synthesis and characterization of metal-ion adsorptive properties of partially carboxymethylated cellulose Carbohydrate Polymers 1998;37:409-414). A schematic outline of the reaction is given in Figure 1A.

Fourier transformed infrared (FTIR) spectroscopy are used in the attenuated total reflectance (ATR) mode to assure the substitutions of carboxymethyl groups at the amino group as well as the primary hydroxyl sites of the chitosan (FTIR-ATR; Varian 660-IR spectrometer with Golden Gate ATR auxiliary) (Chen SC, Wu YC, Mi FL, Lin YH, Yu LC, Sung HW. A novel pH-sensitive hydrogel composed of N,O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. J Control Release 2004;96:285-300). Dried powder of sample is placed onto the ATR crystal directly. The spectra are acquired at 4000-750 cm\(^{-1}\) wave numbers with a 4 cm\(^{-1}\) resolution.

**Preparation of O-carboxymethyl chitosan**
O-carboxymethyl chitosan (O-CMC) can be prepared by reacting monochloroacetic acid with chitosan in isopropanol/NaOH solution using state-of-the-art procedures (e.g. Upadhyaya L, Singh J, Agarwal V, Tewari RP. Biomedical applications of carboxymethyl chitosans. Carbohydr Polym 2013;91:452-466).

Preparation of N-carboxymethyl chitosan

N-carboxymethyl chitosan (N-CMC) can be obtained by reacting free amino groups of chitosan with glyoxylic acid and subsequent reduction of the resulting aldime with sodium borohydride, as described (e.g. Upadhyaya L, Singh J, Agarwal V, Tewari RP. Biomedical applications of carboxymethyl chitosans. Carbohydr Polym 2013;91:452-466).

Printing of N,O-CMC-polyphosphate layers and tissue-like-blocks

N,O-CMC is sterilized, for example, by ultraviolet radiation (254 nm) overnight. Then a solution of 60 mg/ml of N,O-CMC is prepared in physiological saline. After stirring until being homogenous the gel is supplemented with solid Na-polyP until the concentration of 20 mg/ml is reached. The hydrogel preparation formed is completed with 60 mg/ml sodium alginate (e.g., W201502 from Sigma-Aldrich) and stirred at 50°C until it becomes homogenous. Then this hydrogel is filled into sterile printing cartridges (e.g., 30 ml printing cartridges from Nordson EFD) and centrifuged for 3 min at 1500 rpm to remove remaining air bubbles. After connecting the 0.25 mm tapered polyethylene printing tip (Nordson EFD) the cartridge is placed into the preheated (25°C) printing head of the 3D-bioplotter; for example, a 3D-Bioplotter, 4th generation bloter, from Envisiontec can be used.

Bioprinting is performed following described procedures (Neufurth M, Wang XH, Schröder HC, Feng QL, Diehl-Seifert B, Ziebart T, Steffen R, Wang SF and Müller WEG. Engineering a morphogenetically active hydrogel for bioprinting of bioartificial tissue derived from human osteoblast-like SaOS-2 cells. Biomaterials 2014; DOI: 10.1016/j.biomaterials.2014.07.002; in press). The printing solution, composed of 60 mg/ml of N,O-CMC, 60 mg/ml of alginate and 20 mg/ml of Na-polyP is prepared at 25°C using a pressure of 1.4 bar and a printing speed of 18 mm/s. The pre-flow is set to 0.15 s whereas the post-flow amounts to -0.05 s. Cylindrical scaffolds measuring 50 x 0.4 mm are designed, sliced and transferred to the printer software as described (Neufurth M, Wang XH, Schröder HC, Feng QL, Diehl-Seifert B, Ziebart T, Steffen R, Wang SF and Müller WEG. Engineering a morphogenetically active hydrogel for bioprinting of bioartificial tissue derived from human osteoblast-like SaOS-2 cells. Biomaterials 2014; DOI: 10.1016/j.biomaterials.2014.07.002; in press). The strand distance between the printed cylinders is set to 1 mm, resulting in a pore size of the printed layers/block of approximately 0.5 x 0.5 mm. Those scaffolds, layers/blocks, are printed directly into sterile 94 mm Petri dishes, supplemented with 1% [w/v] CaCl₂ as crosslinking solution (Schloßmacher U, Schröder HC, Wang XH, Feng Q, Diehl-Seifert B, Neumann S, Trautwein A, Müller WEG. Alginate/silica composite hydrogel as a potential morphogenetically active scaffold for three-dimensional tissue engineering. RSC Advances 2013;3:11185-11194). After a ≈2 min incubation period the CaCl₂-solution is drained and the cross-linked scaffolds produced are washed twice with distilled water and once with 70% ethanol. The printing of a two layered scaffold with 5 cm in diameter lasts approximately 6 min. The size of the scaffold samples for the cell culture experiments is 20 mm [diameter] x 0.4 mm [thickness].

In the example, a tissue-like block is printed after analysis of the cranial defect, a pig underjaw has been selected, by microtomography [μCT]. The implant dimensions to be printed are predetermined using the computer program Bioplotter RP 2.9 CAD software (Envisiontec). Using the same software, the cylinders are subsequently sliced to individual
layers corresponding to the diameter of the printing needle and subsequently transferred to the VisualMachines 3.0.193 printer software (Envisiontec).

**Preparation of \( N.O\)-CMC hydrogel layer and \( N.O\)-CMC-polyP-PEC**


The polyelectrolyte complex (PEC) (Mi FL, Shyu SS, Wong TB, Jang SF, Lee ST, Lu KT. Chitosan-polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using triplyphosphate or polyphosphate reagent. J Appl Polymer Sci 1999;74:1093-107) is prepared from chitosan powder (60 mg/ml) and Na-polyP (20 mg/ml) as described (Ong SY, Wu J, Moochhala SM, Tan MH, Lu J. Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. Biomaterials 2008;29:4323-4332). The sample is termed “Chitosan-polyP”.

**Scanning electron microscopy and energy-dispersive X-ray spectroscopy**

The scanning electron microscope (SEM; HITACHI SU 8000) is coupled to an XFlash 5010 detector, an X-ray detector that allows simultaneous energy-dispersive X-ray (EDX)-based elemental analyses. This is coupled at voltage of 4 kV to the XFlash 5010 detector that is used for element analysis. HyperMap databases are collected, as described (Salge T, Terborg R. EDS microanalysis with the silicon drift detector (CDD): innovative analysis options for mineralogical and material science application. Anadolu Univ J Sci Technol 2009;10:45-55).

**Determination of the hardness of the \( N.O\)-CMC-polyP scaffold**

The hardness of the scaffolds can be determined, for example, by a ferruled optical fiber-based nanoindenter as described (Chavan D, Andres D, Iannuzzi D. Note: ferrule-top atomic force microscope. II. Imaging in tapping mode and at low temperature. Rev Sci Instrum. Apr 2011;82(4):046107; doi: 10.1063/1.3579496; Chavan D, van de Watering TC, Grucu G, Rector JH, Heeck K, Slaman M, Iannuzzi D. Ferrule-top nanoindenter: an optomechanical fiber sensor for nanoindentation. Rev Sci Instrum 2012;83:115110; doi: 10.1063/1.4766959). The indents are depth controlled (10 μm) and the loading and unloading period is set to 2 s. Based on the load-displacement curves the reduced Young's modulus [RedYM] is calculated.

**Light microscopic analyses**

Digital light microscopic studies can be performed, for example, using a VHX-600 Digital Microscope (Keyence) equipped with a VH-Z25 zoom lens.

**Mineralization by cells in vitro on chitosan matrices**

For example, human osteogenic sarcoma cells, SaOS-2 cells can be used. The cells are cultivated in McCoy’s medium in a humidified incubator at 37°C and 5% CO2 (Wiens M, Wang XH, Schröder HC, Kolb U, Schloßmacher U, Ushijima H, Müller WEG. The role of biosilica in the osteoprotegerin/RANKL ratio in human osteoblast-like cells. Biomaterials 2010a;31:7716-7725). Culture medium/fetal calf serum [FCS] is changed every 3 d. Where
mentioned the cells are exposed to the osteogenic cocktail [OC], containing 10 nM
dexamethasone, 5 mM β-glycerophosphate and 50 mM ascorbic acid. The scaffold samples
20 mm [diameter] x 0.4 mm [thickness] are placed to the bottom of the 24–well plates.

The extent of mineralization can be assayed, for example, by Alizarin Red S and measured
spectrophotometrically (Wiens M, Wang XH, Schloßmacher U, Lieberwirth I, Glasser G,
Calcif Tissue Intern 2010b;87:513-524). Prior to the measurement the chitosan matrices are
removed from the 40-well plates. The amount of bound Alizarin Red S is expressed in nmoles
and correlated to total DNA in the samples.

Effect of blood clotting time
The influence of the N,O-CMC matrices, with and without polyP, on blood clotting time can
be determined, for example, by the assay described by (Shih MF, Shau MD, Chang MY,
Chiou SK, Chang JK, Cherg JY. Platelet adsorption and hemolytic properties of liquid
crystal/composite polymers. Int J Pharm 2006;327:117-125). The samples (100 to 150 mg)
are submersed in bottles placed in a thermostated water bath at 37°C for 10 min. Then 300 μl
of human blood sample (acid-citrate-dextrose with 20 μl/ml of 100 mM CaCl₂) is dropped on
the surface of the matrices until they are completely covered. Then the assays are continued to
be incubated (37°C) for 10 min. Then 15 ml of distilled water are added without disturbing
the clotted blood. Subsequently 10 ml aliquots are taken, centrifuged (100×g; 30 s) and the
supernatant is collected and the clotting test is performed spectrophotometrically at 542 nm.

Statistical analysis
The results can be statistically evaluated using paired Student’s t-test.
CLAIMS

1. A method for the preparation of a scaffold for tissue engineering and repair, comprising the steps of
   i) combining carboxymethyl chitosan, polyphosphate, and alginate,
   ii) three-dimensional (3D) printing (bioprinting) of the resulting hydrogel and
   iii) hardening of the material after printing by exposure to calcium ions.

2. The method according to claim 1, wherein said carboxymethyl chitosan has been formed by carboxymethylation of the amino groups of chitosan (N-carboxymethyl chitosan) or the hydroxy groups of chitosan (O-carboxymethyl chitosan), or both (N,O-carboxymethyl chitosan).

3. The method according to claims 1 and 2, wherein non-carboxymethylated (free) amino groups or hydroxy groups, or both, of said carboxymethyl chitosan are acetylated or partially acetylated.

4. The method according to any of claims 1 to 3, wherein said polyphosphate and the alginate are present as a sodium salt.

5. The method according to any of claims 1 to 4, wherein the average chain length of the polyphosphate is between 10 and 100 phosphate units.

6. The method according to claim 5, wherein the average chain length of the polyphosphate is about 40 phosphate units.

7. The method according to any of claims 1 to 6, wherein said alginate has been supplemented with gelatin or another collagen-derived product.

8. The method according to any of claims 1 to 7, wherein said hydrogel as formed is supplemented with an additional morphogenetically active oligomer or polymer.

9. The method according to claim 8, wherein said additional morphogenetically active polymer is polymeric silicic acid (silica) or one of its salts.

10. The method according to claim 9, wherein said polymeric silicic acid has been formed by an enzyme or protein involved in biosilica (amorphous, hydrated silicon oxide) metabolism, such as, for example, silicatein or a silicatein fusion protein or combinations thereof.

11. The method according to claim 10, wherein a silicatein or a silicatein fusion protein or combinations thereof, as well as a suitable substrate are present.

12. The method according to claims 10 and 11, wherein said silicatein polypeptide or silicatein fusion protein has been produced using a prokaryotic or eukaryotic expression system, or has been produced synthetically.

13. The method according to any of claims 1 to 12, wherein said hydrogel is supplemented with bioglass (bioactive glass) (nano)particles composed of SiO₂·CaO·P₂O₅ or SiO₂·Na₂O·CaO·P₂O₅ of various molar ratios, for example SiO₂·CaO·P₂O₅ of a molar ratio
(mol.%) of 55:40:5 or SiO₂:Na₂O:CaO:P₂O₅ of a molar ratio (mol.%) of 46.1:24.4:26.9:2.6 (45S5 Bioglass®).

14. The method according to any of claims 1 to 13, wherein said hydrogel is simultaneously printed with a suspension of bioglass (bioactive glass) (nano)particles using a three-dimensional (3D) printing technique (two-component scaffold).

15. The method according to any of claims 1 to 14, wherein cells are suspended in said hydrogel and the resulting cell-containing hydrogel is subjected to 3D printing (bioprinting) and subsequent hardening by exposure to calcium ions, wherein said cells are no human embryonic stem cells.

16. The method according to claim 15, wherein said cells are bone-forming cells or bone-dissolving cells or their precursors, or a mixture of both.

17. A 3D-bioprinted scaffold, obtained by a method according to any of claims 1 to 16.

18. The 3D-bioprinted scaffold according to claim 17, wherein said scaffold is in the form of bone implant material or a part of such material.

19. The 3D-bioprinted scaffold according to claim 17, wherein said scaffold is in the form of customized implant fabricated by 3D printing, 3D cell printing (bioprinting), or another rapid prototyping/solid free-form fabrication process.

20. The bone implant material according to claim 18 or 19 for use in the treatment of bone defects.
**Patents Act 1977: Search Report under Section 17**

**Documents considered to be relevant:**

<table>
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<th>Category</th>
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<td>Marine Drugs, 12 (2), 21 February 2014, Wang et al, &quot;The marine sponge-derived inorganic polymers, biosilica and polyphosphate, as morphogenetically active matrices/scaffolds for the differentiation of human multipotent stromal cells...&quot;, 1131-1147 (see abstract and discussion)</td>
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**Categories:**

- **X** Document indicating lack of novelty or inventive step
- **Y** Document indicating lack of inventive step if combined with one or more other documents of same category.
- **&** Member of the same patent family
- **A** Document indicating technological background and/or state of the art.
- **P** Document published on or after the declared priority date but before the filing date of this invention.
- **E** Patent document published on or after, but with priority date earlier than, the filing date of this application.

**Field of Search:**

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC:

Worldwide search of patent documents classified in the following areas of the IPC:

A61L

The following online and other databases have been used in the preparation of this search report:

WPI, EPODOC, TXTE, TXTT, TXTCNT, TXTCNS, TXTKRT, BIOSIS, MEDLINE
**International Classification:**

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