Biomimetic Mineralization of Collagen by Combined Fibril Assembly and Calcium Phosphate Formation

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To find new ways for the synthesis of improved bone implant materials, we studied the mineralization of collagen in vitro. Collagen was mineralized by combining the collagen fibril assembly and the formation of calcium phosphate in one process step. Both reactions were initiated simultaneously by mixing an acid, calcium-containing collagen solution with a phosphate-containing neutralization buffer. Under suitable conditions first fibril assembly occurred along with the precipitation of an amorphous calcium phosphate phase. Subsequently, the amorphous calcium phosphate transformed into a crystalline, apatite-like phase, as revealed by IR spectroscopy and X-ray diffraction. In this way, a homogeneously mineralized collagen gel was obtained, consisting of a three-dimensional network of collagen fibrils covered with calcium phosphate. The attachment between the collagen fibrils and the calcium phosphate crystals could be further improved by the addition of polyaspartate to the reaction mixture. In the absence of polyaspartate the calcium phosphate crystals formed clusters loosely bound to the fibrils, while in its presence separate crystals were located on or inside the collagen fibrils. The applied method is useful for studying the mineralization of collagen and offers a promising approach for the development of new bone implant materials.

Introduction

The mineralization of collagen in vitro is of great interest for the understanding of the mechanisms underlying the mineralization in vivo as well as for the synthesis of improved bone grafts. The substitution of bone is a frequent issue in medicine. Currently autogenous bone, i.e., bone which is taken from another place of the same patient, is the best available material for this application.[2] Obviously, it is desirable to find an artificial material for this purpose. Our aim is to develop a synthetic material which is as similar as possible to natural bone. Since collagen and calcium phosphate are the major constituents of human bone, bone implant materials consisting of these substances have been developed.[3,4]

To achieve properties that are similar to the properties of bone, it is necessary to mimic not only the composition of the natural bone but also its structure. The structure of bone has been revealed down to the structure of the mineralized fibril,[5−7] though some questions remain on the organization on the molecular level. Up to now, it has not been possible to produce a material in vitro with a structure similar to the structure of bone. Our goal is to approach such a structure by biomimetic synthesis of the collagen−calcium phosphate composite. In the first step we have focused our effort on the preparation of mineralized collagen fibrils. Biomimetic synthesis includes synthesis in aqueous solution at physiologic pH, temperature, and ionic strength, but does not stop at this point. In agreement with the biomimetic concept, the general feature of the processes occurring in the living organism during bone growth has been used as guidance. Learning from nature, we intended to study the essential mechanisms of the formation of a bonelike hydroxyapatite−collagen composite.

It is known that mineralization in vivo is controlled by some proteins which are found in bone, the so-called noncollagenous proteins.[8] Gerstenfeld et al.[9] could show through in vitro studies of tissue formation with chicken osteoblast cultures that bone formation can be considered as a multistage process. First a collagen template is grown, and then noncollagenous proteins are formed, which finally control the crystallization of the calcium phosphate. The noncollagenous proteins contain unusual amounts of anionic amino acids (aspartate, glutamate). For this reason polymers of these amino acids, polyaspartate and polyglutamate, have been used as model compounds for the noncollagenous proteins.

The interaction of polyaspartate and polyglutamate with calcium has been investigated in a number of studies. It has been shown that polyaspartate and

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polyglutamate just as the noncollagenous proteins have a strong effect on the kinetics of calcium phosphate formation.[10,11] Poly-L-glutamate has been shown to delay both amorphous calcium phosphate formation[12] and amorphous−crystalline transformation.[13] For a general review on the effect of noncollagenous proteins on mineralization in vitro, see the paper by Boskey.[14]

Very few papers have been published about the effect of anionic polyelectrolytes or proteins on the mineralization of collagen in vitro, i.e., in experimental systems where collagen *and* calcium phosphate are present. Hunter et al.[15] studied the effect of chondroitin sulfate on the mineralization of a collagen gel. In some studies gelatin (denatured collagen) was used instead of collagen.[16] Most studies are restricted to the interaction of polyelectrolytes or proteins with collagen *or* calcium phosphate. However, as will be shown, some of the effects of polyaspartate will only occur in a system containing collagen *and* calcium phosphate. Consequently, it is the purpose of this work to study the interaction of collagen, calcium phosphate, and polyaspartate.

In the past decade, a number of studies on the biomineralization of calcium phosphate have been performed in the double diffusion system, where the counterions are diffusing from different sides through a gel.[16,17] The mineralization in gels has the advantage of the absence of convection. Furthermore, mechanical impact on the collagen and the calcium phosphate crystallites can be avoided in contrast to a stirred system.[18] However, the necessity of diffusion of ions into the gel leads to spatially and temporally changing concentrations and to the mineralization of only parts of the gel. For the use as bone graft a homogeneously mineralized collagen is needed. Therefore, we developed a new method, wherein the calcium and phosphate components have been added to the collagen solution before the sol−gel transformation.

Experimental Section

Materials. Acid soluble collagen type I from calf skin (Fluka) was used as obtained. Sodium poly-L-aspartate was achieved from Sigma (P5387, Lot 64H5526). The weight-average molecular weight obtained by viscosity was 13 700 as stated by the manufacturer, corresponding to a chain length of about 100 residues.

Mineralization of Collagen Fibrils. Collagen was dissolved at a concentration of 1 mg/mL in 10 mM hydrochloric acid at a temperature of 4 °C.

For concentrations of 9.0 mmol/L Ca and 5.4 mmol/L P the following procedure was used: A calcium-containing collagen solution and a phosphate-containing neutralization buffer were prepared separately at a temperature of 4 °C. The calcium-containing collagen solution was made by mixing 108 μL of an aqueous solution of $CaCl_2$ (0.1 M) and 600 μL of a collagen solution. The neutralization buffer was prepared by mixing 160 μL of tris(hydroxymethyl)aminomethane (0.5 M, pH 7.4 adjusted with HCl), 110 μL of NaCl (2 M), 21.6 μL of a KH_2PO_4/K_2HPO_4 buffer (0.5 M, pH 7.4), and 528 μL H_2O.

The reaction was started by adding 492 μL of the neutralization buffer to the collagen solution. The final concentrations were 0.5 mg/mL collagen, 9 mmol/L $CaCl_2$, 5.4 mmol/L phosphate, 110 mmol/L NaCl, and 40 mmol/L tris(hydroxymethyl)aminomethane. After mixing, the reaction mixture was poured into a prewarmed photometer cuvette; in this way the reaction temperature of 30 °C was reached within a few minutes. The pH after mixing was 6.8.

For other calcium phosphate concentrations corresponding volumes of calcium and phosphate solutions and of H_2O were used. If polyaspartate was applied, it was included in the neutralization buffer. For the calcium phosphate precipitation 10 mM HCl was used instead of the collagen solution.

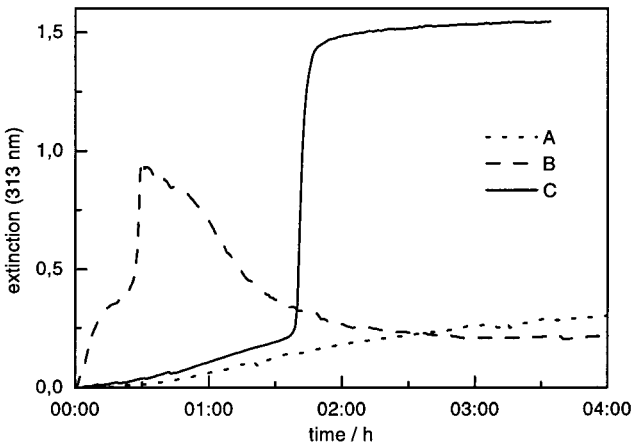

Figure 1. Turbidity during fibril assembly (A), calcium phosphate precipitation (B), and combined fibril formation and mineralization (C). The reactions were monitored through the extinction at 313 nm. The extinction at the start of the reactions was set to zero.

Collagen Fibril Formation. The fibril formation in Figure 1 was carried out under the same conditions as described above, but without phosphate to avoid calcium phosphate formation. For studying the effect of polyaspartate on the kinetics of the collagen fibril formation, the following procedure was applied: Collagen was dissolved at a concentration of 0.4 mg/mL in 10 mM hydrochloric acid at 4 °C. Fibril formation was induced by mixing with an equal amount of a neutralization buffer with the following composition: 260 mmol/L NaCl, 40 mmol/L KH_2PO_4/K_2HPO_4 buffer (pH 7.4). Polyaspartate was included in the neutralization buffer at the appropriate concentration. The collagen solution and the neutralization buffer were maintained at the assembly temperature (30 °C) for 30 min before mixing.

Turbidity Measurement. For turbidity measurements the reaction mixture was poured into a photometer cuvette immediately after mixing. The cuvette was placed in a photometer (ATI Unicam 5625) with a thermostated cuvette holder. The extinction was monitored at 313 nm.

Reproducibility of the Kinetic Measurements. As pointed out in the following, the kinetics of the experiments, especially the combined fibril formation and mineralization, are very sensitive to the experimental parameters (calcium and phosphate concentration (Figure 2), but also the pH). This indicates that a slight change in the experimental conditions can cause variations in the absolute curves. All experiments within one figure are made within one series of experiments, with the same stock solutions. In this case we obtained a good reproducibility as indicated by the two curves in Figure 2. Though variations in the absolute values may be larger between different series of experiments, all principal effects have been reproduced.

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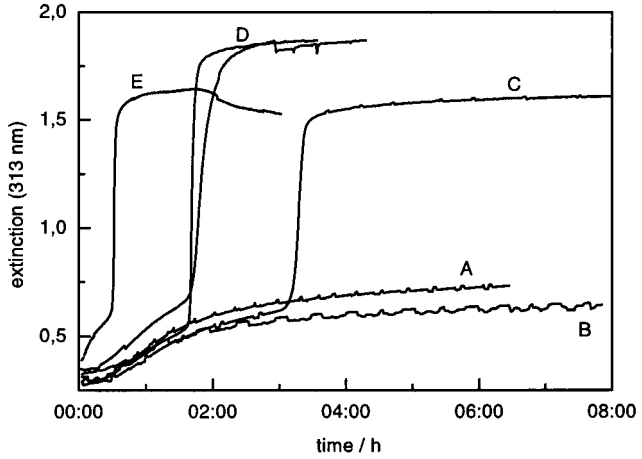

Figure 2. Effect of the calcium phosphate concentration on the kinetics of combined fibril assembly and mineralization. The reaction was monitored by measuring the extinction at 313 nm over time. Concentrations were (A) 7.5 mmol/L Ca, 4.5 mmol/L P, (B) 8 mmol/L Ca, 4.8 mmol/L P, (C) 8.5 mmol/L Ca, 5.1 mmol/L P, (D) 9 mmol/L Ca, 5.4 mmol/L P, and (E) 10 mmol/L Ca, 6 mmol/L P. In case D to curves are printed two indicate the reproducibility of the experiments.

Scanning Electron Microscopy. The samples were prepared by taking some microliters out of the mineralized collagen gel with a pipet and depositing it onto a silicon substrate. The mineralized collagen was allowed to settle for about 1 min, and the fluid was sucked up with a filter paper. Buffer salts were removed by setting a drop of deionized water onto the sample and sucking it up (two times). The samples were air-dried. Scanning electron microscopy (SEM) was performed with a DSM 982 Gemini (Zeiss).

Scanning Force Microscopy. Scanning force microscopy (SFM) was performed with a Nanoscope IIIa (Digital Instruments). Samples were prepared in the same way as the samples for scanning electron microscopy.

Infrared Spectroscopy. For IR spectroscopy the mineralized collagen gel was centrifuged (10 min, 1000*g*) and suspended in deionized water to remove the buffer salts. The centrifugation/suspension cycle was repeated three times. After the last suspension the samples were frozen at −80 °C and freeze-dried. The IR spectra were recorded with an FTIR spectrometer Type Nicolet 510A in KBr.

X-ray Diffraction. The sample preparation was identical with the sample preparation for IR spectroscopy. The diffraction measurements were performed with a STOE transmission diffractometer. The samples were mounted between Mylar foils. Co Kα1 radiation was applied using a Ge(111) primary monochromator.

Results

Combined Fibril Formation and Mineralization. Collagen is, if not cross-linked, soluble at low pH and can be reconstituted to fibrils by neutralization, which can be performed by mixing with a neutral buffer solution. The fibril formation is associated with a strong increase of the viscosity of the solution and leads, under appropriate conditions, to a three-dimensional network of collagen fibrils with gellike properties.[19] The course of the process can be monitored easily by the increase of the turbidity of the solution; the turbidity−time plot has a sigmoidal shape (Figure 1, curve A).

The turbidity change during the precipitation of calcium phosphate from aqueous solution is shown in curve B. The formation of calcium phosphate from aqueous solution and its observation by turbidity have been investigated in detail by Termine and Eanes.[20] Our observations follow their findings; the interpretation of the curve is as follows: The turbidity increases in the first step due to the precipitation of an amorphous phase and reaches an intermediate plateau value. After about 30 min, the transformation to a crystalline phase causes a second, very rapid increase of the turbidity. In contrast to the observations of Termine and Eanes, in our case this increase is terminated suddenly, and the turbidity decreases due to the sedimentation of the calcium phosphate, because we are working in an unstirred system. The very sudden start of the sedimentation indicates that the rapid increase of the turbidity is caused by a fast growth and/or aggregation of the crystallites, and not by an increase of the number of crystallites.

By mixing an acid, calcium-containing collagen solution with a phosphate-containing neutralization buffer, the two reactions were combined in one process step. In this way, both reactions were initiated simultaneously. Because the collagen fibrils are supposed to act as templates for the mineralization, it is necessary that the fibril formation takes place before the precipitation of the calcium phosphate.

Under appropriate conditions the desired sequence of events can indeed be obtained. Curve C shows the turbidity during an experiment carried out in the mentioned way. After mixing, the pH was 6.8. The first part of the turbidity−time plot is similar to the fibril formation curve. After about 100 min, the turbidity rises rapidly, however, and leads to a second plateau value.

The reaction is a combination of fibril and calcium phosphate formation, and a superposition of the turbidity curves A and B would indeed yield a curve like the observed one. The formation of the collagen fibrils and the simultaneous formation of the amorphous calcium phosphate phase would lead to a smooth increase of the turbidity. Subsequently the transformation of the amorphous calcium phosphate to a crystalline phase would cause a rapid rise in turbidity. In contrast to the formation of calcium phosphate in aqueous solution (curve B), no precipitation occurs, because the reaction mixture becomes more and more gellike with the formation of the collagen fibrils.

Whereas the amorphous−crystalline transformation is clearly detectable by its strong increase of the turbidity, the formation of the amorphous phase cannot with certainty be concluded from the turbidity measurements, because it is overlaid by the fibril formation. However, it is well-known that the formation of hydroxyapatite is preceded by an amorphous phase under the applied conditions.[21,22] To additionally prove this, we took samples before the strong rise in turbidity, in which we were able to detect the presence of calcium phosphate by IR spectroscopy and the presence of very small calcium phosphate particles by scanning electron microscopy.

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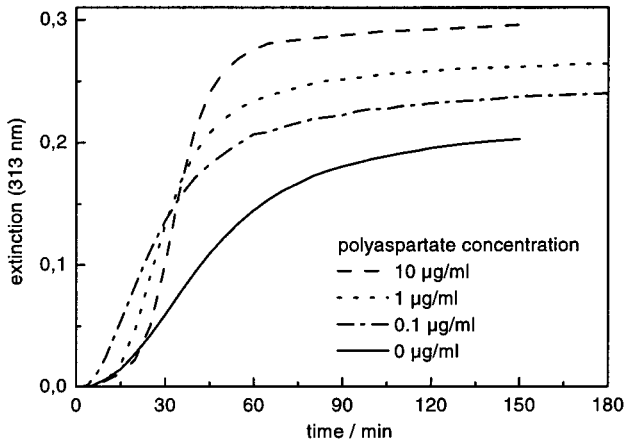

Figure 3. Effect of polyaspartate on the kinetics of collagen fibril formation.

This leads to the following interpretation of the turbidity−time curve: The first, smooth increase is caused by the fibril assembly and the simultaneous formation of an amorphous calcium phosphate phase. Subsequently the amorphous calcium phosphate phase transforms very fast into a crystalline phase, which results in a rapid rise of the turbidity.

As can be seen in Figure 1, the amorphous−crystalline transformation is retarded in the presence of collagen in comparison to the calcium phosphate formation without collagen.

Effect of Calcium Phosphate Concentration. The required sequence of the reactions can only be achieved in a narrow window of solution conditions. Results of experiments with different concentrations of calcium phosphate are shown in Figure 2. The experiment discussed in the previous section (Figure 1, curve C) at concentrations of 9.0 mmol/L calcium and 5.4 mmol/L phosphate is shown in this plot as curve D. Lowering the calcium and phosphate concentrations leads to a similar turbidity−time plot, however, as expected, to slower calcium phosphate formation (curve C, 8.5 mmol/L calcium, 5.1 mmol/L phosphate). When the concentrations are further decreased (curve B, 8.0 mmol/L calcium, 4.8 mmol/L phosphate), the turbidity−time plot has the form of the first part of Curve D, but no rapid rise in turbidity occurs, even after several days. Under these conditions, no precipitation of calcium phosphate could be detected and no precipitated mineral is visible by scanning electron microscopy. It has to be noted that the solution is highly supersaturated with respect to hydroxyapatite under these conditions.

On the other hand, if the calcium and phosphate concentrations are increased a small amount (curve E, 10 mmol/L Ca, 6 mmol/L P), the rapid rise in turbidity occurs a few minutes after mixing. This time is not sufficient enough for the collagen to form fibrils, which is necessary because the fibrils should act as templates for the mineralization. In this case, the process leads not to a gel, but to an apparently structureless precipitate.

Hence, a decrease of about 10% from the concentrations allowing successful mineralization of collagen (9 mmol/L Ca, 5.4 mmol/L P) leads to a complete suppression of mineralization, while an increase of the same order leads to immediate precipitation of calcium phosphate.

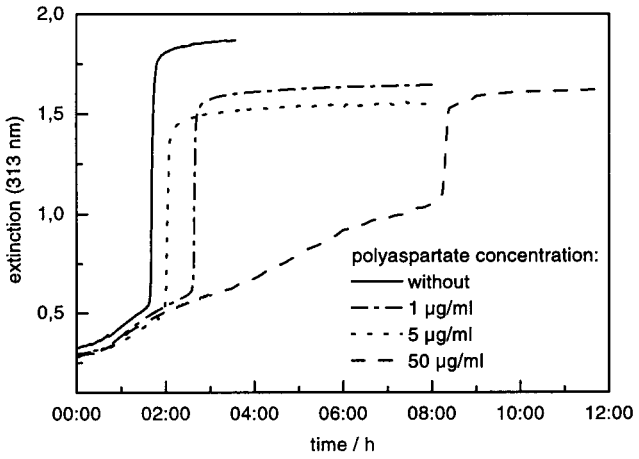

Figure 4. Effect of polyaspartate on the kinetics of combined fibril formation and mineralization.

Effect of Polyaspartate on Fibril Formation. The effect of polyaspartate on the kinetics of the collagen fibril formation was studied in a series of experiments where the fibril formation was carried out in the presence of different concentrations of this polyamino acid (Figure 3). The collagen fibril formation is strongly affected by polyaspartate at concentrations as low as 0.1 μg/mL, which corresponds to 0.05 wt % and 1.5 mol % of the collagen concentration. The extinction in the plateau phase rises in the presence of polyaspartate; the increase is nearly proportional to the logarithm of the polyaspartate concentration. The effect on the kinetics of the fibril formation is not so unambiguous. At low concentrations (0.1 μg/mL) the polyaspartate clearly accelerates the fibril formation, while at higher concentrations the polyaspartate delays the fibril formation with respect to the experiment with 0.1 μg/mL polyaspartate.[23]

Effect of Polyaspartate on Mineralization Kinetics. The effect of polyaspartate on the kinetics of the collagen mineralization has also been studied (Figure 4). The experiments were performed as described above with the polyaspartate incorporated in the neutralization buffer before mixing. The kinetics of the mineralization is strongly affected by the presence of polyaspartate. Whereas in its absence the amorphous−crystalline transformation occurs after about 100 min, it takes place after 8 h in the presence of 50 μg/mL polyaspartate. During the delay of the amorphous−crystalline transformation, the turbidity increases further. This may be caused by ongoing precipitation of amorphous calcium phosphate.

Microscopy. The product of the mineralization process described above is a gel consisting of a three-dimensional network of collagen fibrils, on which crystals of calcium phosphate have settled. Scanning electron micrographs of samples in the absence and in the presence of polyaspartate are shown in Figure 5. In both cases the collagen fibrils are native, showing the typical 67 nm periodicity. Without polyaspartate the mineral crystals form clusters which are loosely bound to the collagen fibrils, while in its presence separate crystals or only small clusters are formed on or in the collagen fibrils.

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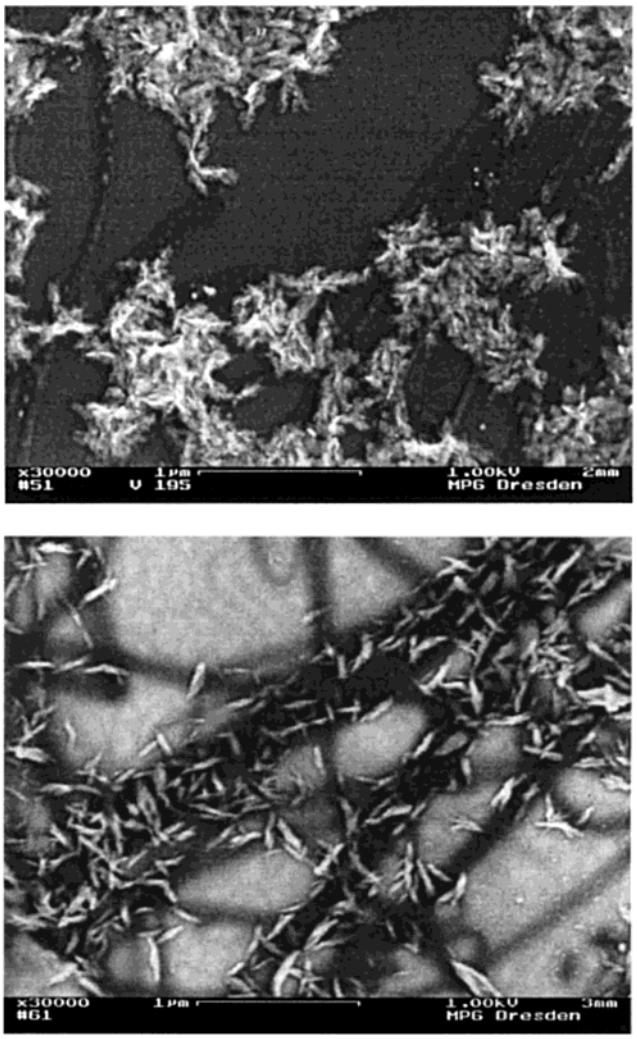

Figure 5. Scanning electron micrographs of mineralized collagen fibrils in the absence (a, top) and the presence, (b, bottom) of 50 μg/mL polyaspartate. The mineralized collagen was transferred to silicon substrates at the end of the reaction. In (a) the silicon substrate was covered with a carbon film, leading to a darker background.

In both cases, the size of the crystals is about 100 nm. However, the morphology of the crystals is affected slightly by the polyaspartate. While in the absence of polyaspartate the crystals show a more platelike shape, in its presence they have a more needlelike morphology. In the presence of polyaspartate, some of the crystals seem to be inside the collagen fibrils. A preferred orientation of the crystals parallel and perpendicular to the collagen fibrils is observed.

We also used SFM to image the mineralized collagen fibrils. With SFM a higher resolution can be obtained in contrast to SEM. In Figure 6 a SFM image of collagen fibrils mineralized in the presence of 50 μg/mL polyaspartate is shown. The sample was prepared in the same way as the samples for electron microscopy. In this image, the principal features, which are also visible in the SEM images, can be seen. Needlelike calcium phosphate crystals are attached to the collagen fibrils, and an orientation of the crystals parallel and perpendicular to the fibrils is discernible. In addition, in the SFM images the 67-nm periodicity of the collagen fibrils is clearly visible, indicating that native collagen fibrils were formed. The dissolved collagen is not completely

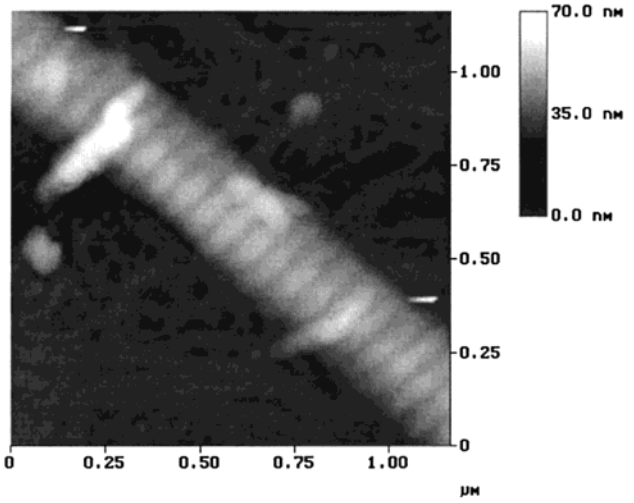

Figure 6. SFM image of a collagen fibril mineralized in the presence of 50 μg/mL polyaspartate (tapping mode, height image).

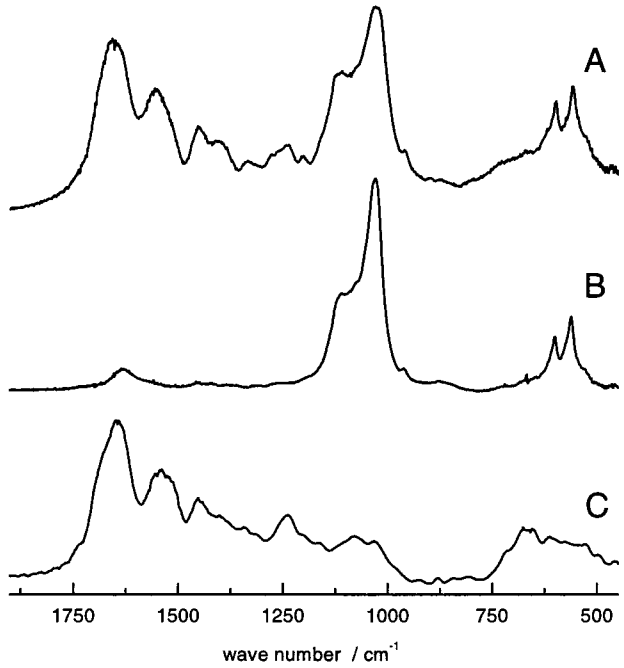

Figure 7. IR spectra of mineralized collagen (A), precipitated calcium phosphate (B), and reconstituted collagen fibrils (C).

transferred to collagen fibrils; microfibrils of incompletely assembled collagen are visible on the surface of the substrate.

Crystallographic Structure. The crystallographic structure of the calcium phosphate was studied with IR spectroscopy and X-ray diffraction. Figure 7 shows IR spectra of mineralized fibrils, calcium phosphate precipitated under the same conditions in the absence of collagen, and reconstituted collagen fibrils. The IR spectrum of the mineralized fibrils appears as a superposition of the spectra of the collagen fibrils and the calcium phosphate. The mineralized collagen fibrils as well as the precipitated calcium phosphate show the typical peaks of the phosphate bands in hydroxyapatite. The peak positions of these bands are reported in Table 1. The positions of the peaks are nearly identical for the mineralized fibrils and the precipitated calcium phosphate but differ slightly from the values for pure

Table 1. Peak Positions of the Phosphate Bands in the IR Spectra of Precipitated Calcium Phosphate, Mineralized Fibrils, and Commercial Hydroxyapatite (HAP) Compared with Values from the Literature for Hydroxyapatite and Octacalcium Phosphate (OCP)[a]

calcium phosphate	mineralized fibrils	HAP (Merck)	HAP[b]	HAP[c]	OCP[c]	assignment
561m	555m	566m	571	561m	559m	ν_4
607m	602m	602m	602	601m	599m	ν_4
		630w	632	631m	630w	OH librational mode
961w	961w	959w	962	962w	962w	ν_1
1027vs	1027vs	1037vs	1050	1040vs	1025s	ν_3
					1035s	ν_3
					1055s	ν_3
					1075s	ν_3
1115s	1115s	1094s	1089	1092s	1105s	ν_3

[a] The wavenumber in cm^{-1} is given. [b] Leung et al.[29] [c] Fowler et al.[24]

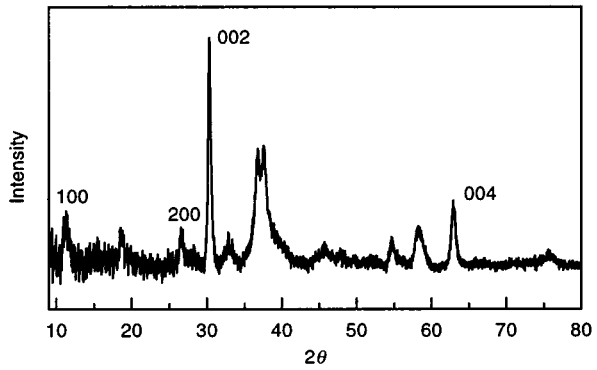

Figure 8. X-ray diffraction pattern of mineralized collagen fibrils (Co Kα1 radiation). The Miller indices refer to hydroxyapatite. Only peaks discussed in the text are labeled.

hydroxyapatite. Especially the ν_3 bands between 1000 and 1100 cm^{-1} are closer to the positions of octacalcium phosphate as observed by Fowler et al.[24] than to the position of hydroxyapatite. On the other hand, none of the additional ν_3 bands reported by Fowler et al. for octacalcium phosphate could be observed.

In the spectrum of the precipitated calcium phosphate, weak bands of carbonate appear at 1450 and 870 cm^{-1}. In the spectrum of the mineralized fibrils, these bands are concealed by bands of collagen. However, it is reasonable that the crystals of the mineralized fibrils also contain carbonate, since no precautions were made to exclude carbon dioxide.

The X-ray diffraction pattern of the mineralized collagen fibrils is shown in Figure 8. The diffraction pattern meets roughly, but not exactly, the diffraction pattern of hydroxyapatite. As has been shown by Brown et al.,[25] hydroxyapatite is capable of forming interlayered crystals with octacalcium phosphate. These crystals consist of layers of hydroxyapatite and octacalcium phosphate parallel to the *bc* plane and joined epitaxially in this plane. If the layers are small enough, combination peaks are observed instead of the individual peaks of the two phases.

As reported in Table 2, the observed diffraction peaks of the mineralized collagen fibrils indeed are situated between the positions expected for hydroxyapatite and those observed for octacalcium phosphate. The peak at 11.3° corresponds to a *d* spacing of 9.10 Å. This is between the d_{100} of hydroxyapatite at 8.17 Å and the corresponding d_{200} of octacalcium phosphate at 9.34 Å. The peak at 26.6° (3.89 Å) is also located between the positions expected for the (200) peak of hydroxyapatite (4.08 Å) and the (500) peak of octacalcium phosphate (3.74 Å). This indicates the presence of interlayers of octacalcium phosphate in the hydroxyapatite.

Table 2. Diffraction Peaks and the Resulting *d* Spacings of Figure 8 Compared with the Values of Pure Hydroxyapatite and Octacalcium Phosphate[a]

mineralized fibrils		HAP		OCP	
2θ	d/Å	d/Å	*hkl*	d/Å	*hkl*
11.285	9.10	8.168	(100)	9.343	(200)
15.184	6.77			6.23	(300)
18.545	5.55	5.263	(101)	5.519	(1$\bar{1}$1)
26.611	3.89	4.084	(200)	3.74	(5$\bar{0}$0)
27.955	3.70	3.89	(111)		
30.329	3.42	3.44	(002)	3.425	(002)
32.794	3.17	3.171	(102)		
36.813	2.83	2.817	(211)	2.837	(4$\bar{1}$2)
37.634	2.77	2.723	(300)	2.669	(7$\bar{0}$0)
39.65	2.64	2.631	(202)		
45.7	2.30	2.265	(310)		
47.985	2.20	2.152	(311)	2.215	(8$\bar{3}$0)
54.572	1.95	1.945	(222)		
55.513	1.92	1.892	(312)		
58.202	1.84	1.841	(213)		
62.899	1.71	1.72	(004)		
75.678	1.46				

[a] Calculated from their unit cell parameters. Combination peaks indicating the interlayering of hydroxyapatite with octacalcium phosphate are printed bold.

The intensities of the peaks differ also from the values of pure hydroxyapatite. Especially the peaks at 30.3° (002) and at 62.9° (004), originated from the lattice planes perpendicular to the *c* axis of the crystals, are too strong compared with the other diffraction peaks. This is due to a preparation texture of the sample. The mineralized collagen gel has no texture, but a texture may be induced by mounting the collagen fibrils between the Mylar foils. In this way, a preferred orientation of the calcium phosphate crystals with regard to the collagen fibrils could be converted to an overall texture.

Discussion

To overcome the unsatisfactory performance of current bone implant materials, we investigated new ways for the synthesis of collagen−calcium phosphate composites. We started from the assumption that a material similar in composition and structure to natural bone will be a good implant material, and that such a material can be achieved by using biomimetic pathways. To

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approach the structure of natural bone, we regard the in vitro mineralization of collagen fibrils as the first step for the assembly of a bonelike composite. Though under investigation for a long time, it had not been made to resemble the structure of in vivo mineralized fibrils by in vitro mineralization until now.

Combined Fibril Formation and Mineralization. Here we describe a new method for the in vitro mineralization of collagen. This method makes use of the advantages of gel-based crystallization, but overcomes the inhomogeneous mineralization caused by the diffusion of the ionic components into the gel by incorporating the ions into the collagen solution before sol−gel transformation. Fibril assembly and calcium phosphate precipitation are initiated simultaneously by mixing an acid, calcium-containing collagen solution with a phosphate-containing neutralization buffer. Since the collagen fibrils are supposed to act as templates for the mineralization, it is nevertheless necessary that the fibril formation is completed to a large extent before the precipitation of the calcium phosphate occurs. This could be reached by accurate tuning of the experimental conditions and happens only in a closely restricted range of calcium phosphate concentration. By this method a homogeneously mineralized collagen gel could be obtained. Under suitable conditions, first collagen fibrils are formed simultaneously with an amorphous calcium phosphate phase. Subsequently, the amorphous calcium phosphate is transformed to a crystalline phase.

It has been under discussion for decades whether collagen acts as a promoter or inhibitor of the mineralization; however, recent studies point more in the direction that collagen has no effect on the mineralization.[14] From our experiments it cannot be decided whether the retardation of the amorphous−crystalline transformation in the presence of collagen (Figure 1) is a specific effect of the collagen; it could be an effect of the physicochemical properties of the collagen gel compared with the aqueous solution.

The formed calcium phosphate is a defect apatite with interlayers of octacalcium phosphate, which is similar to the phase precipitated under equal conditions in the absence of collagen. The defect structure and the small crystal size of about 100 nm should give this calcium phosphate a higher solubility compared to stoichiometric hydroxyapatite and make it similar to bone apatite. Applied as bone replacement material, it should therefore have a good remodeling capability.

Kinetic Effects of Polyaspartate. Polyaspartate affects the kinetics of the fibril assembly as well as the kinetics of the calcium phosphate formation. The effect on the fibril assembly is complex and can be accelerating as well as delaying depending on the applied concentration of the polyamino acid. In the case of the mineralization, only delaying effects could be observed in this study. Mainly the amorphous−crystalline transformation of the calcium phosphate is affected. Whether polyaspartate also affects the formation of the amorphous phase cannot be concluded with certainty. However, the ongoing increase of turbidity before the amorphous−crystalline transformation points to the fact that the formation of the amorphous phase continues while the transformation to the crystalline phase is delayed. The kinetic effect on the mineralization is far stronger than the effect on the fibril formation.

Polyaspartate interacts with calcium phosphate through adsorption to the surface of the particles.[26] The delay of the calcium phosphate crystallization may be due to interaction with the amorphous calcium phosphate (retardation of the dissolution) or due to interaction with the crystalline phase by inhibition of the growth of nuclei.

A delay of calcium phosphate formation through polyaspartate has been observed before,[10] but in an agarose gel instead of collagen gel. Kinetic arguments are often used to classify biopolymers as inhibitors or promotors of mineralization. However, substances that delay mineralization under some conditions promote the mineralization under others. Furthermore, the delay of mineralization does not automatically mean "worse" mineralization, since a decrease in reaction rates often leads to an increase in selectivity.

Structural Effects of Polyaspartate. Investigations of the structure of the mineralized collagen fibrils should give further insight. The applied method leads to a mineralization on the level of individual collagen fibrils. Whereas the calcium phosphate crystals tend to form clusters in the absence of polyaspartate, in its presence only separated crystals or small clusters grow which are bonded to the collagen fibrils. The attachment of the calcium phosphate crystals is clearly improved by the addition of polyaspartate, which may be caused by electrostatic interaction. Polyaspartate adsorbs to the surface of calcium phosphate crystals[26] and is known to be a good dispersant.[27] The charged surfaces may then cause repulsion between the negatively charged crystallites and attraction between crystallites and the positively charged collagen fibrils.

In the presence of polyaspartate some crystals seem to be inside the collagen fibrils; this is not observed in the absence of polyaspartate. This may be due to nucleation by polyaspartate which is incorporated into the collagen fibril; however, it may also be due to incorporation of already nucleated crystals into the forming fibrils, since the fibrillogenesis is not terminated when the amorphous to crystalline transformation takes place. The processes overlap. In the case of calcium carbonate, it has been observed that polyaspartate drives the mineralization into gelatin films, but on a much larger dimension.[28,29]

Conclusions

The effect of polyaspartate on calcium phosphate formation has been investigated in a number of studies as a model compound for noncollagenous proteins, which contain an exceptional amount of this amino acid. Though it is believed that noncollagenous proteins and other biopolymers are responsible for the control of the mineralization of collagen, few studies have been published until now about the interaction of these substances on the mineralization of collagen in vitro. No

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study has been published about the interaction of collagen, calcium phosphate, and polyaspartate.

In our study it could be demonstrated that polyaspartate improves the connection between calcium phosphate and collagen. Such effects can only be observed in the presence of polyaspartate, collagen, *and* calcium phosphate. Though this increases the complexity of the system and the number of possible interactions, it is therefore desirable that more experiments are carried out regarding the effect of a substance on the mineralization of collagen in vitro.

The method described is suitable for investigations in this direction. Compared with the double diffusion system the method presented has the advantage of spatially homogeneous concentrations, which makes the analysis of the system easier. However, the process is more complex due to a temporal change of the mineralization matrix and the sensitivity to experimental conditions.

Further work is in process to build composite materials by incorporating the mineralized fibrils in a calcium phosphate cement. In these materials the presence of polyaspartate can improve the connection between the mineralized collagen fibrils and the calcium phosphate cement.

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