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Precipitation of carbonateapatite from a revised simulated body fluid in the presence of glucose

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ABSTRACT: *Revised simulated body fluid (rSBF) was modified by the addition of glucose in a physiological amount. The influence of this compound on calcium phosphate crystallization from supersaturated solutions equal to 4 x rSBF ionic concentrations was studied under physiological conditions (solution pH=7.35-7.40, temperature 37.0 ± 0.5 °C). The experiments were performed in both plastic vessels (fast-uncontrolled precipitation) and in a constant-composition double-diffusion (CCDD) device (slow precipitation under strictly controlled conditions). Solutions used had different concentrations of hydrogencarbonate ions and with or without Hepes buffer. Regardless of the experimental conditions chosen, glucose was found to have a negligible influence on calcium phosphate crystallization from rSBF, while hydrogencarbonate ions had a strong influence on the structure and chemical composition of the precipitates. (Journal of Applied Biomaterials & Biomechanics 2003; 1: 200-7)*

KEY WORDS: *Glucose, Hydrogencarbonate, Revised simulated body fluid (rSBF), Calcium phosphates, Crystallization, Biomineralization*

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INTRODUCTION

For mammals, the biological mineralization of bone and teeth is simulated by calcium phosphate precipitation (1, 2). Initially, the precipitation experiments were performed by just mixing aqueous solutions containing calcium and phosphate ions (3-5), but since the introduction of a simulated body fluid (SBF) (6), this medium has become the most popular simulating solution. However, the ionic composition of standard SBF is not equal to that of human blood plasma: chloride concentration is too high, while hydrogencarbonate concentration is too low. Since then, a number of improvements to SBF have been suggested (7, 8) with revised SBF (rSBF) being the most recent (9, 10). However, various SBF modifications contain an artificial buffer (tris (6-8) or Hepes (9, 10)) that is not present in blood plasma. Another important drawback of all SBF types is the absence of the biologically relevant organic compounds (e.g. carbohydrates and proteins) always present in biological liquids (blood, serum, saliva, etc). Presumably, both factors are responsible for the differences seen between the biological apatite in calcified tissues and bone-like apatites precipitated from SBF.

The influence of glucose and other carbohydrates on fluoroapatite (FA) growth (11), on bone mineralization (12), on calcium salt solubility, dental enamel and hydroxyapatite (HA) (13), as well as on enamel dissolution and fluoride uptake from solutions (14) have been studied, being of considerable interest in dentistry. Carbohydrates were found to have a minor influence on HA crystallization (11, 13), but inhibited FA crystallization (11) and bone mineralization (12). The solubility of enamel in buffered sugar solutions is lower at pH 4-6, but similar at pH 7-8 compared to sugar-free buffered solutions (14). The above studies were performed in various buffered solutions (11, 13, 14), but as yet, there are no reported experiments with SBF containing glucose. Therefore, this study aimed to close the gap

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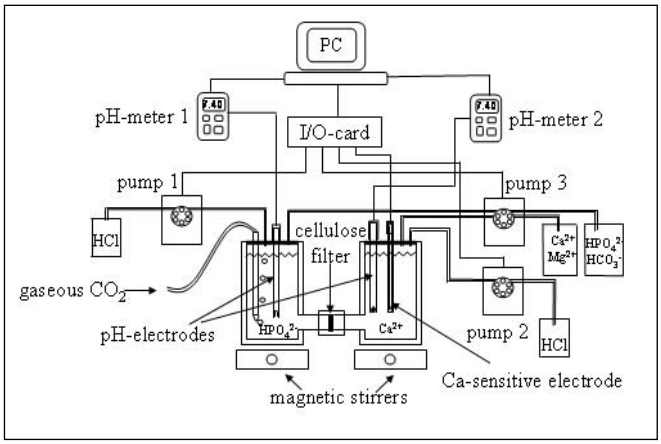


Fig. 1 - ***The principal scheme of the CCDD device. Two pH-meters continuously monitor the solution pH in each vessel. In the right hand vessel, the calcium concentration is continuously monitored by a calcium-selective electrode. To keep the crystallization conditions strictly constant, three peristaltic add the calcium + magnesium and hydrogenphosphate + hydrogencarbonate stock solutions, as well as that of HCl for pH adjustment. Permanent CO_2 bubbling is done in the left hand vessel.***

in the knowledge of glucose influence on calcium phosphate precipitation from SBF.

MATERIALS AND METHODS

The mineralization experiments were performed in both a constant-composition double-diffusion (CCDD) device (Fig. 1) and in plastic vessels. The design and main advantages of the CCDD device have been reported previously (15, 16). Briefly, the CCDD device consists of two thermostatted glass vessels separated by a porous membrane. Each vessel works as an independent constant-composition device, able to keep the solution at a pH 7.35-7.40. Crystallization occurs within pores or on the membrane. The main advantage of this device is the possibility of slow crystallization under strictly controlled solution supersaturation, pH, temperature and hydrodynamics (15, 16).

Of various SBF modifications, we chose rSBF (9, 10) for our study. Aqueous rSBF solutions with and without Hepes buffer were prepared by dissolving the inorganic salts in double-distilled water, as described previously (9, 10). As rSBF is only slightly supersaturated with respect to HA crystallization, precipitation does not occur without promoters. In order to accelerate the precipitation and to increase the amount of precipitates, all experiments were performed with solutions containing 4x the ionic rSBF (4rSBF) concentration. As condensed rSBF solutions never occur in biological systems, this was a serious limitation of our study; however, this experimental approach strongly reduced the precipitation time, which *in vivo* is very slow.

To work with the condensed rSBF solutions, calcium and magnesium cations were separated from hydrogenphosphate and hydrogencarbonate anions by the preparation of two different solutions 4rSBF-Ca and 4rSBF-PO_4, respectively. After mixing in equal amounts 4rSBF-Ca and 4rSBF-PO_4 it became 4rSBF. To eliminate the Hepes buffer, three 4rSBF-PO_4 versions were prepared: one contained Hepes (4rSBF-PO_4-Hepes), whilst the other two did not. For the latter solutions, the pH was adjusted by either HCl (4rSBF-PO_4-HCl) or CO_2 (4rSBF-PO_4-CO_2). To avoid possible bacteria growth during the experiments, we added 0.1 gl^{-1} of NaN_3 to the solutions. Table I summarizes the chemical composi-

TABLE I - ION CONCENTRATIONS OF HUMAN BLOOD PLASMA AND DIFFERENT rSBF SOLUTIONS, mM

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_4^-	HPO_4^{2-}	SO_4^{2-}	pH	NaN_3, g l^{-1}	Hepes-buffer, g l^{-1}
Human blood plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5	7.25-7.40	–	–
Revised SBF (rSBF) (9, 10)	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5	7.40 ± 0.01	–	~ 12
4rSBF-Ca	568.0*	20.0	20.0	12.0	412.0	–	–	2.0	7.40 ± 0.01	0.1	–
4rSBF-PO_4-Hepes	568.0*	20.0	–	–	412.0	216.0	8.0	2.0	7.35 ± 0.03	0.1	~ 80
4rSBF-PO_4-HCl	568.0*	20.0	–	–	~ 460	~ 120	8.0	2.0	7.35 ± 0.03	0.1	–
4rSBF-PO_4-CO_2	568.0*	20.0	–	–	412.0	~ 420	8.0	2.0	7.35 ± 0.03	0.1	–

* – contains extra 1.5 mM of sodium due to the presence of NaN_3

Hepes = 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethane sulfonic acid

tion of the solutions. Glucose, 4 gl^{-1}, was dissolved in all types of 4 rSBF solution. In human blood, the normal glucose concentration is 0.6-1 gl^{-1} (17, 18). Consequently, 4 gl^{-1} corresponds to 4x the normal glucose concentration.

For the experiments performed in the CCDD device (slow crystallization), the 4rSBF-Ca and 4rSBF-PO_4 solutions were placed into different glass vessels (Fig. 1) separated by an inert membrane made of a cellulose filter (ashless 589[3] filter paper, blue ribbon, Schleicher & Schuell, No. 300210). We chose an inert membrane on purpose: at the current stage of our study we did not want to work with any osteointegrative material. Both solutions were in permanent contact inside the pores of the cellulose filter where crystallization occurred under the physiological conditions (T = 37.0 ± 0.5 °C, pH = 7.35 ± 0.05) for 7 days. After each experiment, the filter was removed, washed with deionized water, dried at 37 °C until a constant mass was reached and weighed. A detailed description of the procedure in the CCDD device has been previously reported (15, 16).

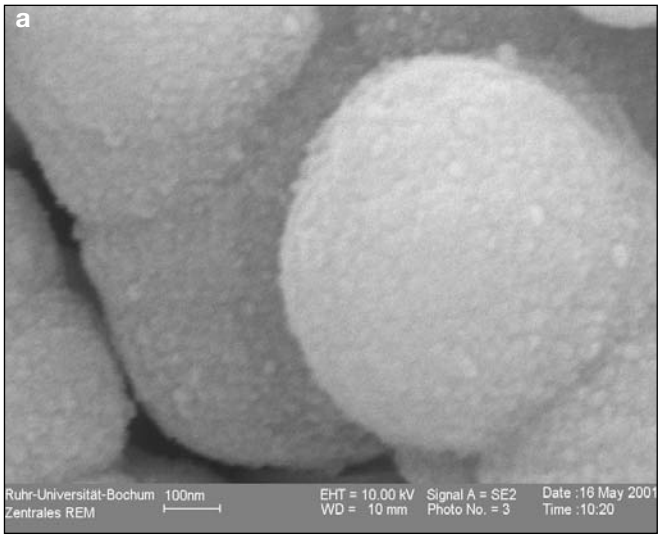
The experiments in the plastic vessels (fast-uncontrolled crystallization) were performed as follows. Preheated (37.0 ± 0.5 °C) of 4rSBF-Ca and 4rSBF-PO_4 solutions (250 ml of each) were mixed in plastic vessels. The vessels were closed immediately and stored at 37.0 ± 0.5 °C for 3 days and periodically shaken. The vessels were then opened and the solution pH measured. The suspensions were filtered, the precipitates washed with deionized water, and dried at 37 °C until a constant mass was reached and then weighed. The final solutions were within pH 7.65-7.80.

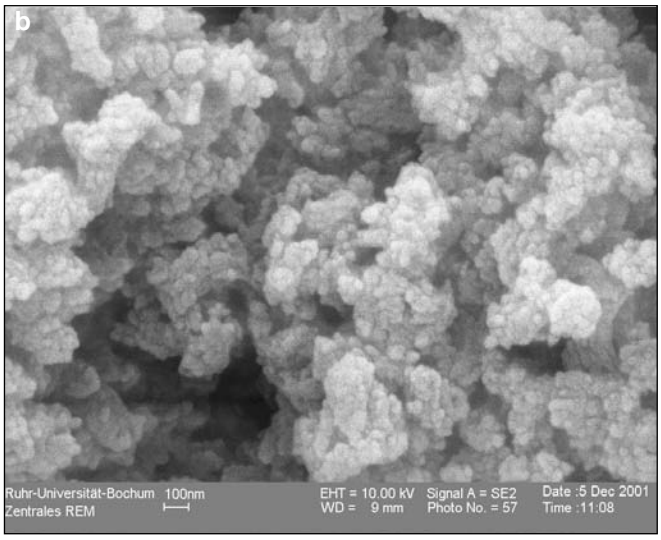
The chemical and structural composition of the precipitates formed in the CCDD device and the plastic vessels was studied by scanning electron microscopy (SEM, LEO 1530, gold sputtering) coupled with energy-dispersive X-ray spectroscopy (EDX) (ISIS; Link Analytical, Oxford Instruments), infrared spectroscopy (IR, 1720X, Perkin Elmer) and X-ray diffraction (XRD, D8 Advance, Bruker AXS; CuKα radiation). For the precipitates formed in the plastic vessels, chemical analysis for calcium, magnesium, phosphate and carbonate was performed. Of each precipitate 0.2 g were dissolved in 10 ml of 0.1 M HCl. The calcium concentration was measured by titration with 0.01 M EDTA at pH=12.0 with calcon carbonic acid (Merck) as an indicator. Magnesium titration was performed with 0.01 M EDTA, but at pH=8.5 with Eriochrom black T (Merck) as an indicator. The phosphate concentration was determined photometrically via the formation of the blue ammonium phosphate-molybdate complex. The amount of carbonate in the solid precipitates was determined by a microdiffusion technique (19). Of the sample 0.02 g were dissolved in 0.5 M $HClO_4$ (in a closed vessel), with evolved CO_2 absorbed by an excess of 0.05 N $Ba(OH)_2$ followed by titration with 0.025 N HCl. The amount of water incorporated was measured gravimetrically by heating up to 300 °C. Unfortunately, the amount of precipitates formed on the cellulose filter in the CCDD device (approximately 10-15 mg) was not enough to determine their chemical composition by a chemical analysis.

RESULTS AND DISCUSSION

SEM pictures of the precipitates formed from 4rSBF in plastic vessels with and without glucose are shown in Figures 2 and 3, and those precipitated in the CCDD device in Figures 4-6. The precipitates rapidly formed in the plastic vessels (Figs. 2, 3) had a different structure compared to those formed slowly under the strictly controlled crystallization conditions (Figs. 4-6). In addition, in two out of three cases, the precipitates formed in the CCDD device had different structures on both sides of the cellulose filter (Figs. 4, 5). Only the precipitates formed from 4rSBF-PO_4-CO_2 were similar on both sides of the filter (Fig. 6). Currently, we cannot explain this; we suggest that the differences in precipitates found on both sides of the cellulose filter are due to cation and anion penetration differences through the filter (e.g. the cellulose filter could have a surface charge influencing ionic penetration).

However, it is clear that the amount of hydrogencarbonate (increasing in "HCl", "HEPES" and "CO_2"; Tab. I), the Hepes buffer presence, and the crystallization kinetics (plastic vessels vs CCDD device) had a strong influence on the precipitates shape and structure. Peculiarities of hydrogencarbonate influence on the crystallization from SBF is found in the references, and the main conclusion is that the precipitates formed under a high hydrogencarbonate ion concentration are less porous and denser than those formed under a low hydrogencarbonate ion concentration (20, 21). This was seen with the precipitates formed in the CCDD device (Figs. 4-6), while for the precipitates formed in the plastic vessels hydrogencarbonate ions influenced only precipitate dimensions (Figs. 2, 3). Therefore, the general influence of hydrogencarbonate also depends on the crystallization kinetics. Concerning glucose influence, the SEM results clearly indicated that, regardless of the experimental conditions and the hydrogencarbonate ion concentration, glucose had a negligible influence on precipitate shape and structure.





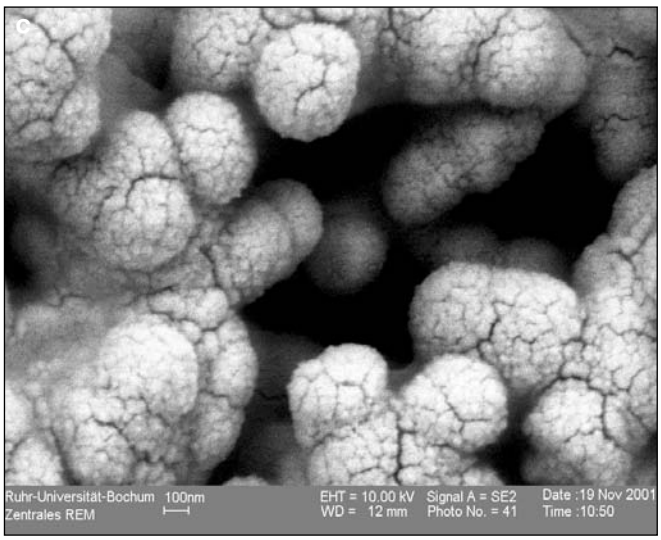
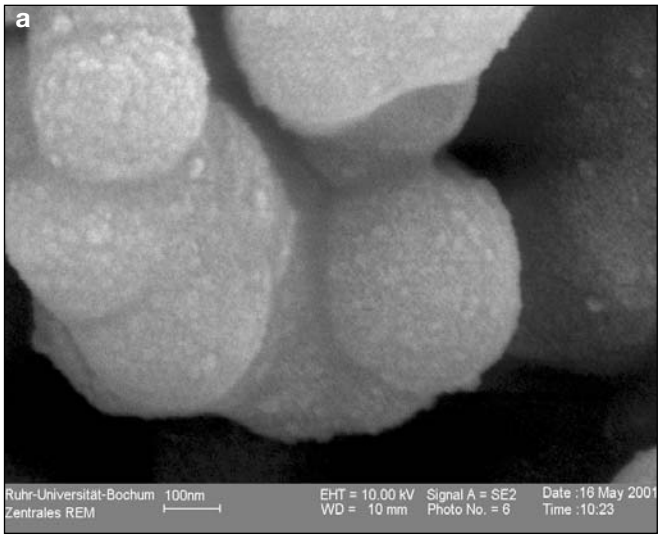
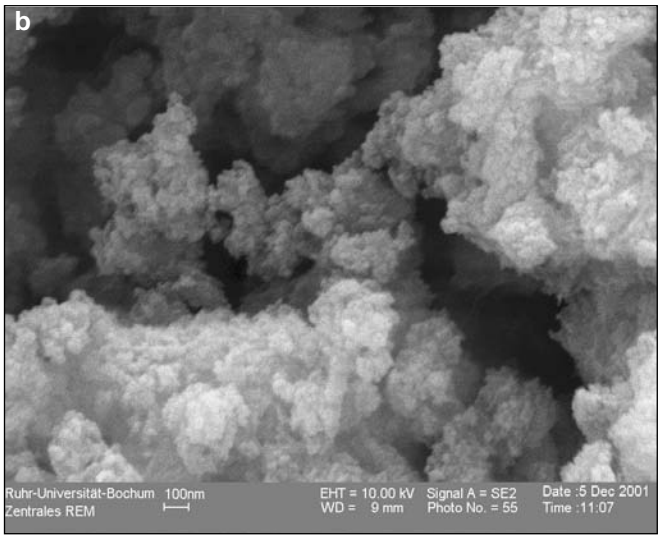


Fig. 2 - *Typical precipitates formed in the plastic vessels from 4rSBF: a) 4rSBF-PO_4-Hepes; b) 4rSBF-PO_4-HCl; c) 4rSBF-PO_4-CO_2. Magnification: 100,000 ×. Bar: 100 nm.*





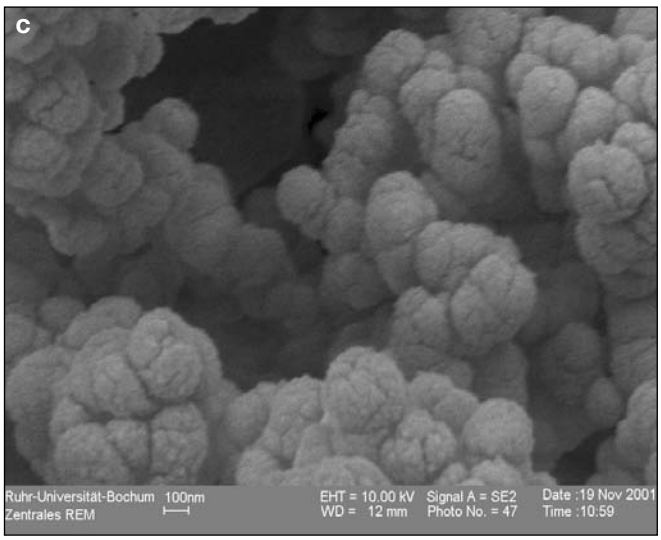


Fig. 3 - *Typical precipitates formed in the plastic vessels from 4rSBF + 4 gl^{-1} glucose: a) 4rSBF-PO_4-Hepes; b) 4rSBF-PO_4-HCl; c) 4rSBF-PO_4-CO_2. Magnification: 100,000 ×. Bar: 100 nm.*

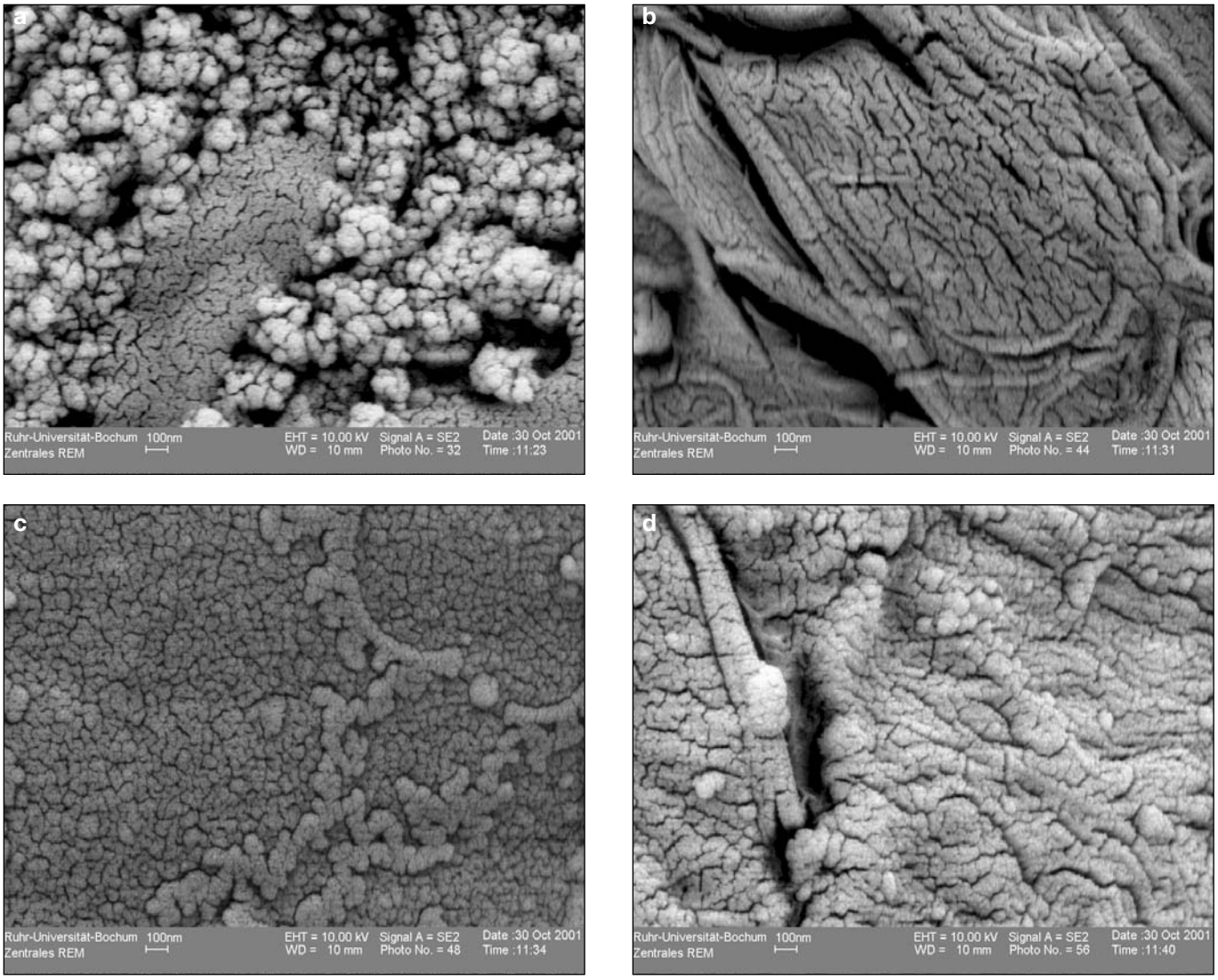


Fig. 4 - ***Typical precipitates formed in the CCDD device from 4rSBF with Hepes: a) calcium side of the filter; b) phosphate side of the filter (both without glucose); c) calcium side of the filter; d) phosphate side of the filter (both with 4 gl^{-1} glucose). Magnification: 100,000 ×. Bar: 100 nm.***

The XRD results of the precipitates formed from 4rSBF in the plastic vessels without glucose are shown in Figure 7. In all cases, the precipitates consisted of a poorly crystallized HA. However, the precipitates formed from the solutions containing the smallest amount of hydrogencarbonate (4rSBF-PO_4-HCl) were always more crystalline than the others. The diffraction patterns of the precipitates formed in the CCDD device (not shown) had negligible differences compared to those precipitated in the plastic vessels. In both cases, the diffraction patterns of the precipitates formed with glucose (not shown) are identical to those formed without glucose. Therefore, the XRD results confirmed the above conclusion regarding a strong influence of hydrogencarbonate concentration and Hepes with a negligible glucose influence.

EDX studies of the precipitates formed from 4rSBF in the plastic vessels with and without glucose revealed that in all cases the precipitates consisted of calcium phosphates. There were only minor differences found between the precipitates formed in the plastic vessels and the CCDD device.

The IR results of the precipitates formed from 4rSBF in the plastic vessels without glucose are shown in Figure 8. The IR spectra of the precipitates formed with glucose (not shown) are indistinguishable from those formed without glucose. As seen in Figure 8, the precipitates are poorly crystallized (absorption bands are poorly resolved). Again, a minor (if any) glucose influence and a strong hydrogencarbonate influence were found. The latter

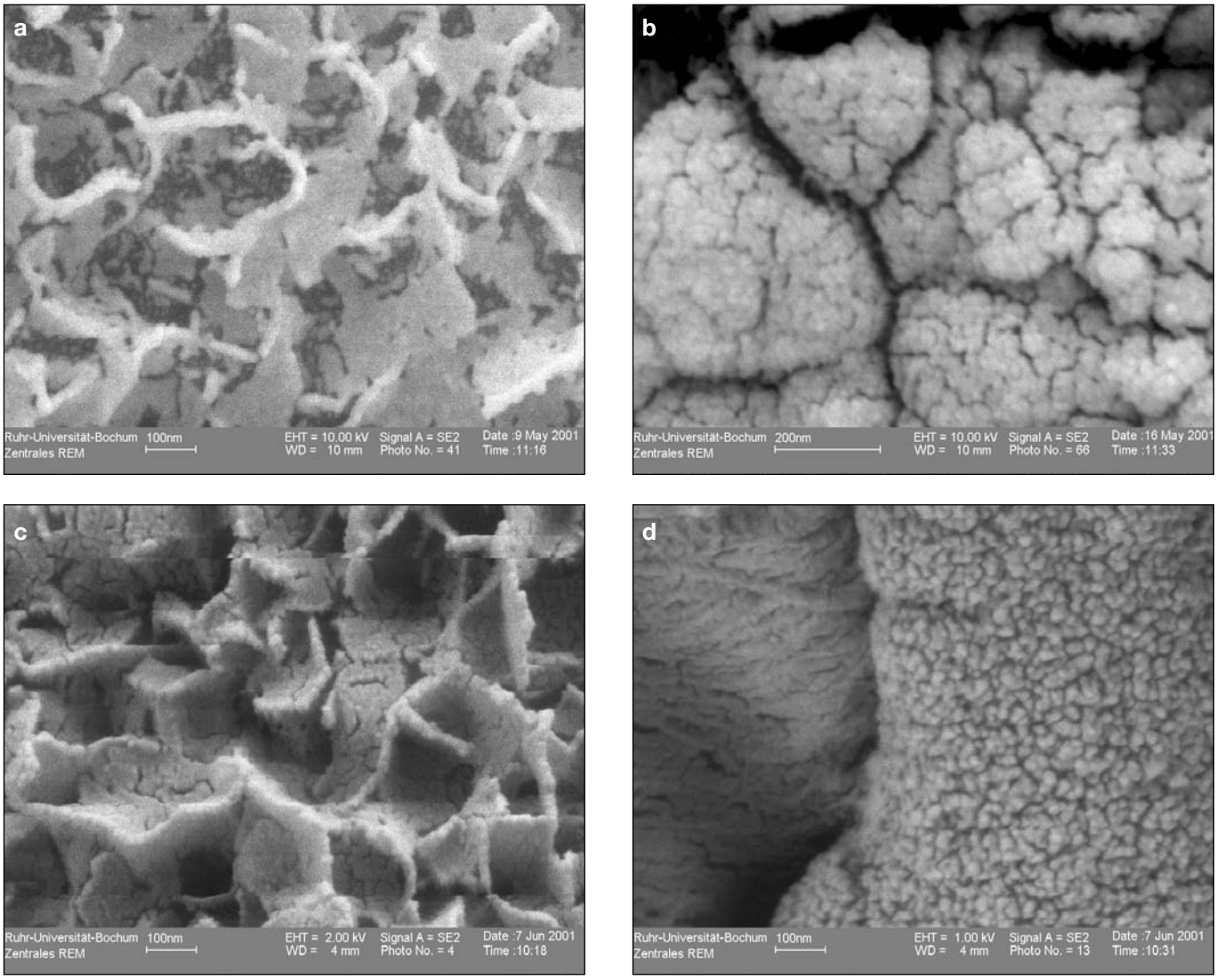


Fig. 5 - *Typical precipitates formed in the CCDD device from 4rSBF with HCl: a) calcium side of the filter; b) phosphate side of the filter (both without glucose); c) calcium side of the filter; d) phosphate side of the filter (both with 4 gl^{-1} glucose). Magnification: 100,000 ×. Bar: 100 nm (a, c, d), 200 nm (b).*

TABLE II -THE CHEMICAL COMPOSITION OF THE PRECIPITATES FORMED FROM 4RSBF IN PLASTIC VESSELS, WT. % BY CHEMICAL ANALYSIS

	Ca^{2+}	Mg^{2+}	$PO4_4^{3-}$	CO_4^{2-}	H_2O	Ca/P (molar)
4rSBF-PO_4-Hepes	30 ± 1	0.5-1	40 ± 3	20 ± 2	10 ± 2	~ 1.8
4rSBF-PO_4-Hepes with 4 gl^{-1} glucose	30 ± 1	0.5-1	39 ± 3	19 ± 2	11 ± 2	~ 1.8
4rSBF-PO_4-HCl	30 ± 1	0.5-1	41 ± 3	17 ± 2	10 ± 2	~ 1.7
4rSBF-PO_4-HCl with 4 gl^{-1} glucose	30 ± 1	0.5-1	42 ± 3	18 ± 2	10 ± 2	~ 1.7
4rSBF-PO_4-CO_2	30 ± 1	0.5-1	38 ± 3	26 ± 2	8 ± 2	~ 1.9
4rSBF-PO_4-CO_2 with 4 gl^{-1} glucose	31 ± 1	0.5-1	37 ± 3	25 ± 2	7 ± 2	~ 1.9

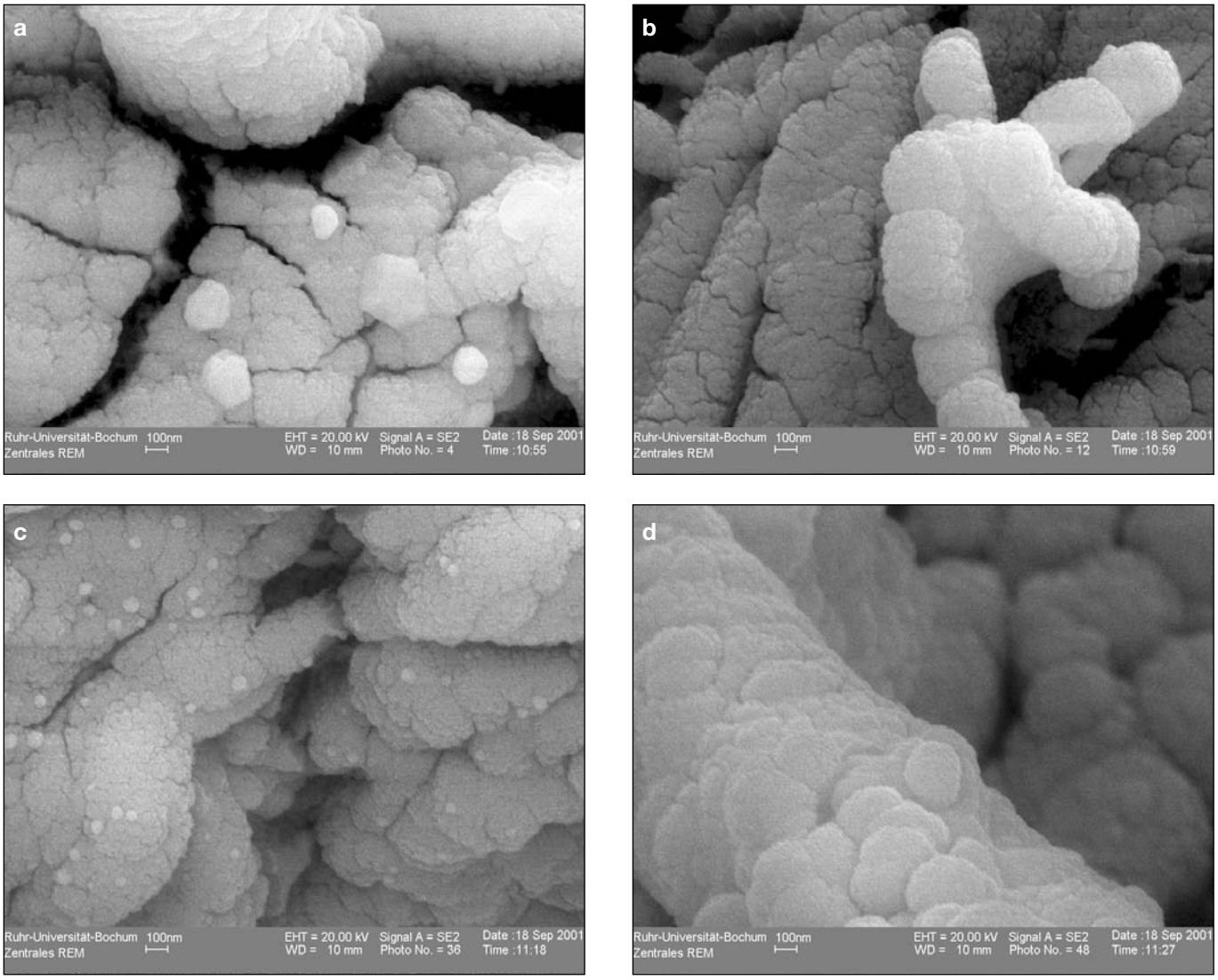


Fig. 6 - *Typical precipitates formed in the CCDD device from 4rSBF with CO_2: a) calcium side of the filter; b) phosphate side of the filter (both without glucose); c) calcium side of the filter; d) phosphate side of the filter (both with 4 gl^{-1} glucose). Magnification: 100,000 ×. Bar: 100 nm.*

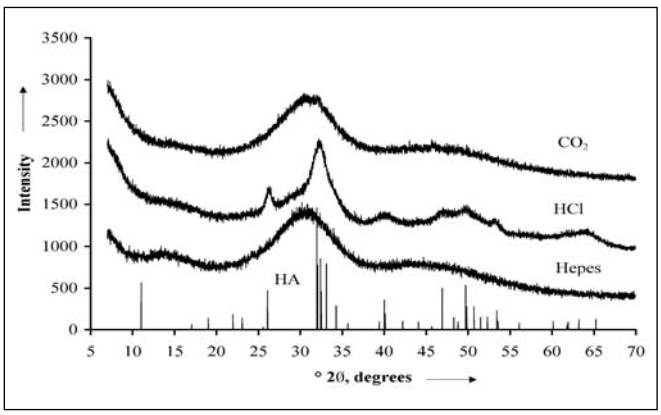


Fig. 7 - *Representative XRD patterns of the precipitates formed in the plastic vessels from 4rSBF without glucose: Hepes - 4rSBF-PO_4-Hepes; HCl - 4rSBF-PO_4-HCl; CO_2 - 4rSBF-PO_4-CO_2; HA - the diffraction pattern of the well crystallized HA.*

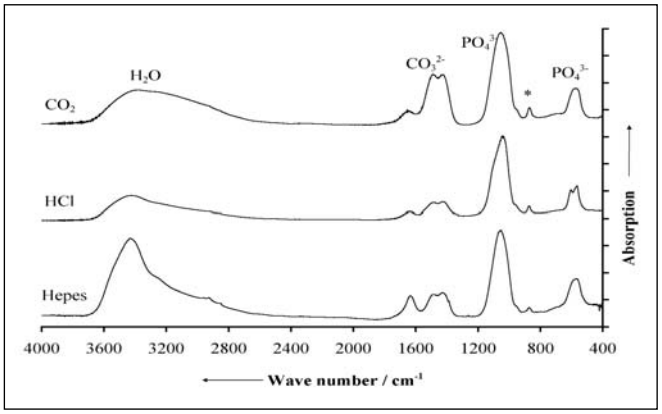


Fig. 8 - *Representative IR spectra of the precipitates formed in the plastic vessels from 4rSBF without glucose: Hepes - 4rSBF-PO_4-Hepes; HCl - 4rSBF-PO_4-HCl; CO_2 - 4rSBF-PO_4-CO_2. The band marked by *corresponds to either CO_3^{2-} or HPO_4^{2-} .*

is seen as intensely increasing the specific absorption bands of carbonate in the range of 1400-1500 cm^{-1}. Table II summarizes the chemical composition of the precipitates formed from 4rSBF in the plastic vessels with and without glucose. An increasing hydrogencarbonate concentration in 4rSBF resulted in phosphate substitution by carbonate (which corresponds with the results of IR spectroscopy), while the calcium and magnesium contents remained constant. Yet again, no glucose influence was found. To conclude, a negligible glucose influence and a very strong hydrogencarbonates influence on calcium phosphate crystallization from aqueous rSBF solutions, we can explain by the well-known ability of carbonates to incorporate into the crystal structure of apatites (3-5), while to our knowledge, there has never been a report on glucose incorporation into the crystal structure of apatites. The latter could be one of the main reasons, why glucose presence does not influence crystallization from rSBF. However, it should be noted that all the results were obtained with condensed rSBF solutions, which never occur in biological systems. This was a serious limitation of this study and additional experiments with standard rSBF solutions are desirable.

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