

Continuous preparation of functionalised calcium phosphate nanoparticles with adjustable crystallinity

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Calcium phosphate nanoparticles can be prepared in almost uniform size and shape by a continuous precipitation process that also allows their functionalisation by organic molecules (DNA, surfactants).

Calcium phosphates are of high relevance in materials science, biology, and medicine because they constitute the inorganic part of human hard tissue, *i.e.* bone and teeth.^{1,2} Except for enamel, calcium phosphate is always nanocrystalline in these structures. Recently, we presented a method to continuously precipitate (unfunctionalised) nanocrystalline calcium phosphate for application as resorbable bone substitution material.^{3,4} The main idea is to precipitate calcium phosphate in a reactor with an overflow that leads to a filter. The solutions of calcium and phosphate are continuously added, and the water from the overflowing solution is immediately removed on the filter, thereby preventing Ostwald ripening of the initially formed nanocrystals. With this method, bulk materials in amounts of up to a few hundred grams per day can be prepared (unfunctionalised).

The precipitates remain nanocrystalline because the water is immediately removed. In order to obtain stable colloidal dispersions, the surface of the particles must be protected against aggregation, *i.e.* a functionalisation agent that adsorbs on the surface is required. Therefore, we added a second reaction vessel to the original setup. The modified crystallisation apparatus is shown in Fig. 1. The precipitate overflows from the first reaction vessel into the second one where a functionalisation agent is added. From this, the colloidal dispersion can be taken, *e.g.* with a syringe.†

The crystallisation parameters (*e.g.*, residence time, temperature) are expected to play a critical role for the properties of the precipitate, *i.e.* mainly its particle size and morphology and its crystallinity. We have varied the crystallisation parameters without functionalising agent and studied their influence on the particle shape and crystallinity. To our surprise, the particles were always spherical and of almost uniform size (Fig. 2). The particle diameter is almost independent of the preparation conditions (Tab. 1). However, the internal order, *i.e.* the crystallinity, depends on the crystallisation conditions in the expected way: the particles that are precipitated at low temperature and short residence time are fully

amorphous, and the particles that are prepared at higher temperature and long residence time are nanocrystalline (data not shown). Crystallographically, the X-ray diffractograms correspond to hydroxyapatite, $\text{Ca}_5(\text{PO}_4)_3\text{OH}$. Broad diffraction peaks are indicative of a poor crystallinity.

These results demonstrate that it is possible to prepare unfunctionalised nanoparticles with almost the same shape (spherical) and size (60 to 90 nm), but with adjustable crystallinity. For an application as resorbable biomaterial, both the particle size and the crystallinity play important roles because each one is directly related to the solubility. Using this method, it is possible to ignore one of the parameters (namely, the crystal size) and to vary only the crystallinity.

By adding suitable organic molecules, functionalised nanoparticles can be kept in solution as a stable colloid. We have tried different compounds, *i.e.* an anionic surfactant (SDS), a cationic

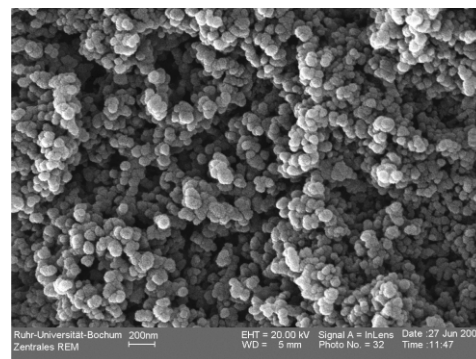


Fig. 2 SEM micrograph of unfunctionalised nanoparticles precipitated at 25 °C with an average residence time of 60 s (see also Table 1).

Table 1 Average particle size in nm of unfunctionalised calcium phosphate nanoparticles precipitated under different conditions (standard deviations given after the average in parentheses: by SEM)

Average residence time	5°C	25°C	50°C	X-ray amorphous
60 s	75±9	83±10	90±9	nanocrystalline
96 s	73±8	75±7	83±6	
175 s	61±11	82±19	85±7	
420 s	65±5	68±9	80±7	

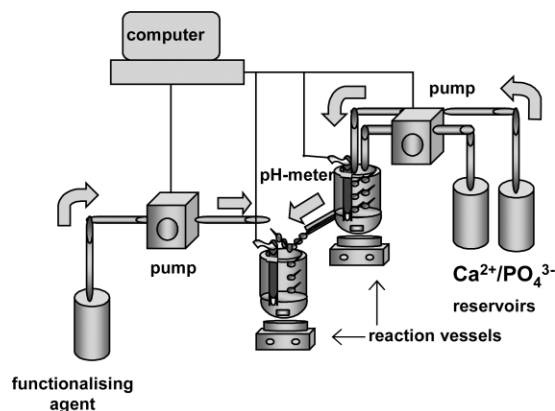


Fig. 1 Schematic setup of the crystallisation and functionalisation apparatus.

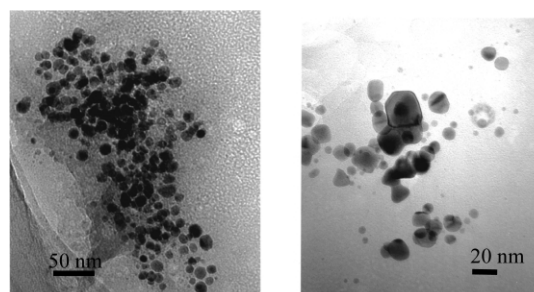


Fig. 3 Transmission electron micrographs of functionalised calcium phosphate nanoparticles that were precipitated in the presence of DNA.

Table 2 Colloid chemical data on functionalised calcium phosphate nanoparticles (standard deviations given after the average in parentheses). All data were recorded 1–2 days after preparation. The light scattering results were repeated and did not change significantly after 7 more days of storage. Note that the values for the zeta potential are also pH dependent

	Calcium phosphate	+ DNA	+SDS	+CTAB	+Triton X-100
pH of dispersion	7.74	9.56	8.76	7.48	7.74
Conductivity/ $\mu\text{S m}^{-1}$	2097	1964	1696	1255	1188
Electrophoretic mobility/ $\text{m}^2\text{V}^{-1}\text{s}^{-1}\cdot 10^6$	+0.52(6)	-2.72(2)	-3.83(11)	+2.40(4)	-0.47(3)
Zeta potential	+7(1)	-37(3)	-52(1)	+33(1)	-6.4(4)
Maxima in light scattering/nm	39	14, 78 (minor)	14–39, 190–250 (minor)	—	280–320, 500–730 (minor)

surfactant (CTAB), a non-ionic surfactant (Triton X-100), and DNA. The dispersion of nanoparticles is mixed rapidly with a solution of the functionalising agent (Fig. 1), thereby preventing further agglomeration of the initially formed particles. In all cases, we obtained stable colloids that did not form precipitates during storage. For characterisation, we used transmission electron microscopy (TEM). Fig. 3 shows a typical TEM micrograph of DNA-coated calcium phosphate nanoparticles. The particle size has decreased to about 10 to 20 nm, a fact that we ascribe to blocking of the surface by the adsorbed DNA. In contrast, we found larger particles when we functionalised with Triton X-100 (several 100 nm).

This different aggregation behaviour is ascribed to differences in the interaction of the functionalising molecules with the calcium phosphate surface. The pure nanoparticles have a positive surface charge, therefore anions (like DNA or SDS) adsorb with high efficiency, as shown by the strongly negative zeta potential (Table 2). However, the positively charged CTAB also strongly adsorbs as shown by the highly positive zeta potential. DNA, SDS, and CTAB all lead to electrostatic stabilisation of the colloid and prevent further aggregation to larger particles. Thus, the average particle size (by light scattering) decreases to about 14 nm from 39 nm for the unfunctionalised particles. The non-ionic surfactant Triton X-100 also adsorbs on the surface but does not prevent aggregation. We therefore conclude that electrostatic stabilisation is more important than steric stabilisation in this system.

A promising application of such well-defined functionalised nanoparticles is the non-viral introduction of DNA into cells, *i.e.* transfection as it is routinely carried out in biochemistry.⁵ There are some reports about similar calcium phosphate nanoparticles in the literature, but the particles are either much larger or they are prepared by a method which is not suitable to incorporate biomolecules: Nanoparticles with incorporated DNA from two microemulsions with particle sizes of about 80 nm;⁶ nanoparticles of calcium phosphate, block-copolymers, and DNA in the size range of 100 nm;⁷ amorphous nanoparticles (about 20 nm diameter) from calcium ethanolate and phosphoric acid in ethanol;⁸ nanoparticles from a hydroxide gel (about 50–20 nm);⁹ nanoparticles with variable chemical composition by high-temperature plasma-spray (10 nm to 4 μm).¹⁰

Because biochemical studies have shown that smaller particles are more effective for gene transfection than larger particles¹¹ and because calcium phosphates themselves are highly biocompatible, we believe that the presented method of preparation has a high potential towards custom-made functionalised nanoparticles for gene transfection. In a preliminary study, we could show that the particles can indeed be used for the transfection of cells.¹²

In conclusion, we have demonstrated how almost monodisperse (unfunctionalised) calcium phosphate nanoparticles with variable crystallinity can be prepared by an easy method. The crystallinity can be adjusted from fully amorphous to nanocrystalline. The stabilisation in form of a colloidal dispersion is possible by adding suitable reagents during the precipitation (functionalisation). While

the non-ionic surfactant Triton X-100 resulted in larger particles, DNA, SDS and CTAB were able to stabilise much smaller particles with about 10 to 20 nm in diameter.

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Notes and references

† For the precipitation reaction, the concentrations of the reagents were $c(\text{Ca}(\text{NO}_3)_2) = 6.75 \text{ mM}$ and $c((\text{NH}_4)_2\text{HPO}_4) = 4.05 \text{ mM}$. The pH of both solutions was adjusted to 10 by KOH before the reaction. The functionalisation was carried out by mixing equal volumes of the resulting dispersion (using nanoparticles precipitated at 25 °C and 175 s residence time) and of the dissolved functionalising agents. As DNA, we used the DNA sodium salt of salmon testes (Sigma; 6.7 mg ml^{-1}), and pcDNA3-EGFP DNA from *E. coli* (80 $\mu\text{g ml}^{-1}$). As surfactants, sodium dodecyl sulfate (SDS; anionic; 6.75 mM; 1.94 mg ml^{-1}), cetyltrimethylammonium bromide (CTAB; hexadecyltrimethylammonium bromide; cationic, 3.375 mM; 1.22 mg ml^{-1}), and Triton X-100 (octylphenoxy polyethoxyethanol; $n = 9$ to 10; non-ionic; 10 mg ml^{-1} ; about 16 mM) were used. The critical micelle concentrations (CMC) at 25 °C are 7.08–8.27 mM (SDS), 0.8–1.0 mM (CTAB), and 230–300 μM (Triton X-100).¹³ In all cases, the concentrations used were either at or above the CMC. All surfactants were obtained from Merck. X-ray powder diffraction data were obtained at beamline B2 at HASYLAB with monochromatic radiation in transmission mode (capillaries) at room temperature. Scanning electron microscopy (SEM) was performed with a LEO 1530 instrument on gold-sputtered samples. Transmission electron microscopy was performed with a Philips CM 200 FEG instrument. Zeta potentials were measured with a ZetaPlus Brookhaven instrument (676 nm laser; Smoluchovsky method). Light scattering was performed with a NICOMP 370 instrument (632.8 nm laser).

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