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Cytotoxicity investigations of plasma sprayed calcium phosphate coatings

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One potential alternative material to replace hydroxyapatite (HAp) as a coating material for plasma-sprayed coatings on implants for hip replacement is fluorapatite (FAp). FAp has advantages over HAp regarding the capability of being chemically stable during the coating process. This leads to surface coatings containing high apatite rates with a mechanical stability (bond strength, microhardness) comparable to HAp. From the technical point of view the production of FAp coatings is well investigated, although studies on biocompatibility of FAp coatings are fewer. This paper reports the production of HAp and FAp coatings with varying solubilities by plasma spraying and their *in vitro* cytotoxicity. Varying solubilities were realized by using modified plasma-spray parameters in common with suitable apatite powders with different crystallinities. Coating solubilities were evaluated by immersing the plasma-sprayed coatings in deionized water and electrolyte solution. Afterwards, cytotoxicity tests were performed using a modified half-slide technique. Cell attachment and cell morphology were evaluated. Neither HAp nor FAp coatings exhibited cytoxic influence on cells in culture. Results suggest that HAp coatings stimulate cell growth and FAp coatings do not. This could be explained by a negative effect on cell growth of the dissolved fluoride ions.

1. Introduction

Depending on their composition, materials show different grades of degradation in their physiological environment. Even ceramic materials, known to be highly resistant to corrosion, are treated in this way [1]. Resulting changes in the biomaterial or loss of material may lead to property changes in the implant and also in the surrounding tissue. For this reason the products of the degradation reactions markedly influence the healing process.

This paper deals with investigations of the solubility of reliable hydroxyapatite and fluorapatite plasma-sprayed titanium. Fluorapatite coatings not only have the advantage of an easier production [2], they are also regarded as being more stable in several solubility tests [3].

Resistance against degradation of apatite coatings may be effected by applying modified plasma-spray parameters, which also includes the use of source powders with different crystallinities. Thus HAp and FAp coatings produced with different parameters are investigated with respect to their *in vitro* behaviour, which is evaluated by extraction in deonized water and immersing in an electrolyte solution.

Finally, cell culture tests investigate the effect of degradation products on cell behaviour in the vicinity of apatite-coated titanium implants.

2. Experimental procedure

2.1. Sample preparation

The investigation methods require that different sample geometries be realized (13 × 50 mm: extraction, immersion; 13 × 15 mm: cell cultures). Thus, small titanium plates (Ti–6Al–4V) were coated with HAp and FAp powders applying atmospheric plasma spraying (APS).

During the plasma spray process the plasma gas stream was created by an electrical arc formed between a finger-type tungsten cathode and a nozzle-type copper anode inside the burner. Plasma gases used were noble gases (argon) or mixtures of diatomic gases (nitrogen, hydrogen). The powder was fed by a powder gas stream into this high energy plasma gas stream. Temperatures in the plasma gas stream may reach 20 000 °C in the centre. The injected powder particles were molten and the material was deposited onto the roughened substrate surface [4]. Depending on the plasma gas composition, the velocity of the molten powder materials during plasma spraying is in

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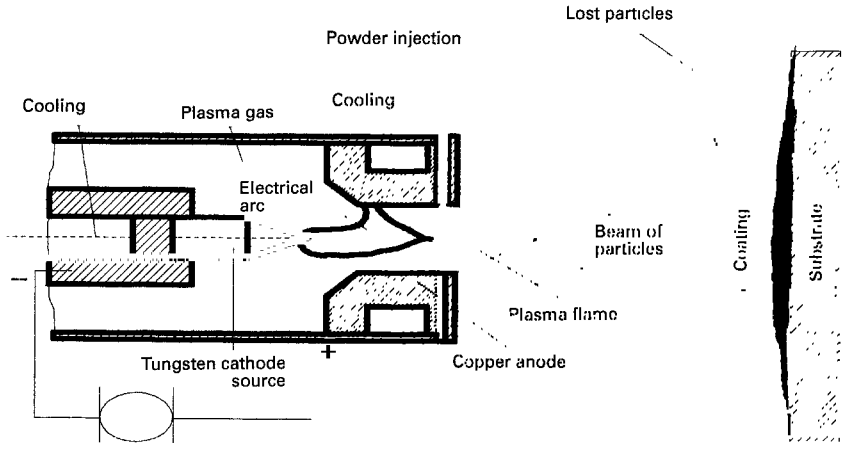

Figure 1 The principle of plasma spraying.

the range 300–800 m s^{-1}. Fig. 1 shows a cross-section of the plasma torch used in an atmospheric plasma spray system and facilitates understanding of the principles of plasma spraying.

For evaluating the influence of plasma spray parameters the plasma gas composition was varied using a mixture of argon, hydrogen and pure nitrogen as gases for the creation of the plasma flame. Plasma power was kept constant during the plasma spray investigations. Another important parameter looked at was the crystallinity of the spray powders used. Hydroxyapatite and fluorapatite powders with different crystallinities (lc-low crystallinity; hc-high crystallinity) were available, both in a particle size distribution between 45 and 125 μm.

2.2. Extraction and immersion of the coated samples

Two different methods were chosen to investigate the various kinds of degradation. In order to determine whether the decomposition is based on solution or erosion the samples were extracted in deionized water. The samples were also immersed in an electrolyte solution to judge changes in the coating structure caused by the ion addition.

The extraction was performed with a Soxhlet apparatus (Fig. 2), which allows maximization of the concentration gradient and diffusion conditions while the washed out material is accumulated in the closed circulation.

Parts of the solvent volume of 450 ml deionized water (pH factor 6.5) condensed on the sample, serving as solution before being changed after about 12 min. Extraction time was 4 weeks. Changes in the immersed coatings and the solvent were examined at intervals of 1 week.

The composition of the applied immersion medium simulated the blood plasma and the extracellular liquid in the human body. The influence of larger, unloaded structures in the physiological environment was not of interest. Solutions were prepared by dissolving the substances sodium chloride, sodium carbonate, potassium chloride, potassium hydrogenphosphate, calcium chloride and magnesium chloride in deionized water [5].

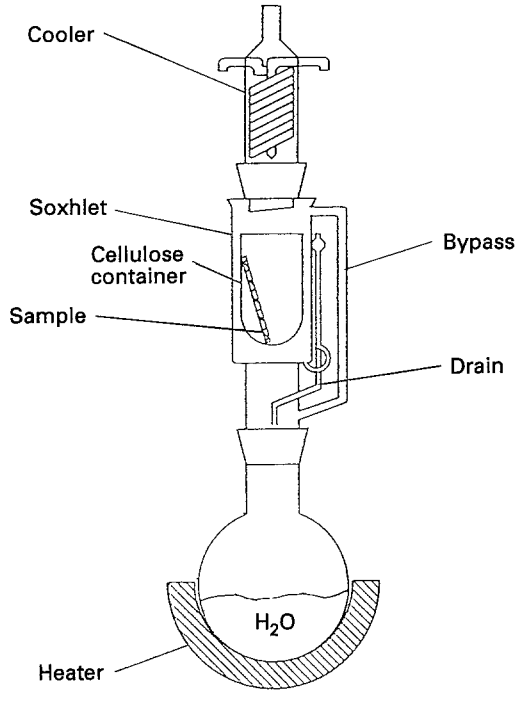

Figure 2 Soxhlet apparatus.

Every coated sample was incubated in a volume of 50 ml at ambient temperature for 1 week. To achieve constant diffusion conditions the samples were kept in constant movement.

2.3. Cell cultures

Cell culture tests were performed to investigate the influence of coating materials or their solubility products on the surrounding tissue. Therefore the cultures were prepared using the half-slide technique [6]. This describes the seeding of cells with direct contact on the coating material and in the vicinity of the sample.

After preparation of the small sample plates they were fixed in the culture dish and the cell suspension was added, containing mouse fibroblasts of the permanent cell line L-929, Dulbecco's modified Eagle

Medium, L-glutamine, penicillin, streptomycin and foetal calf serum. Sample incubation time was 24 h, of a constant 37 °C temperature.

2.4. Evaluation methods

In order to determine material loss caused by erosion the sample weight was observed during the extraction time. Changes in lattice structure and the sample surface were examined by X-ray diffraction (XRD) and scanning electron microscopy (SEM), respectively. Additionally the calcium concentration in every liquid sample was analysed by atomic absorption spectroscopy (AAS) to examine whether there is dissolved calcium after extraction or a change in the concentration after immersion.

After incubation the cell cultures were examined visually by two methods. Cell morphology and cell distribution in the culture dish was evaluated by direct light microscopy and cell vitality by fluorescence microscopy after the addition of a mixture of ethidium bromid and acridine orange.

3. Results

3.1. Dependence of the solubility on the plasma spray parameters

The weighing of the extracted samples and their XRD patterns led to the conclusion that a higher rate of crystallization leads to a lower solubility of the coatings. Fig. 3 shows that the influence of the crystallinity of the source powders is higher than the influence of the plasma gas compositions. Furthermore, the parameter dependence of the coating stability is higher in FAp coatings than in HAp coatings.

SEM-investigations of immersed samples revealed that a process of substance agglomeration on the surface took place, this being even greater than

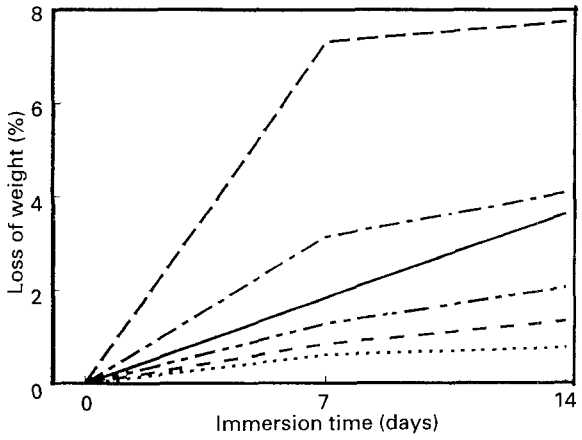

Figure 3 Coating solubility of various HAp and FAp coatings (– – FAp 1, lc powder Ar-H_2 plasma; – · – FAp 2, hc powder, Ar-H_2 plasma; . . . . . FAp 3, hc powder, N_2 plasma; ——— HAp 1, lc powder, Ar-H_2 plasma; – · · – HAp 2, hc powder, Ar-H_2 plasma; – – – HAp 3, hc powder, N_2 plasma).

erosion effects. EDX analyses show that there is only an addition of ions already extant in the coatings. Dependence of calcium absorption on crystallization rate cannot be evaluated by measuring the changes of calcium concentration in the electrolyte solution after incubation. Only the comparison of changes in the liquid samples of FAp and HAp leads to the result that the composition of the ceramic material has an effect.

3.2. Influence of the coating materials on the cell cultures

Both HAp and FAp coatings had good compatability with L-929 fibroplasts. Cells in the vicinity of the HAp and FAp samples had a normal morphology appearance, as also observed using the non-toxic control material Thermanox (Fig. 4). Staining with a mixture

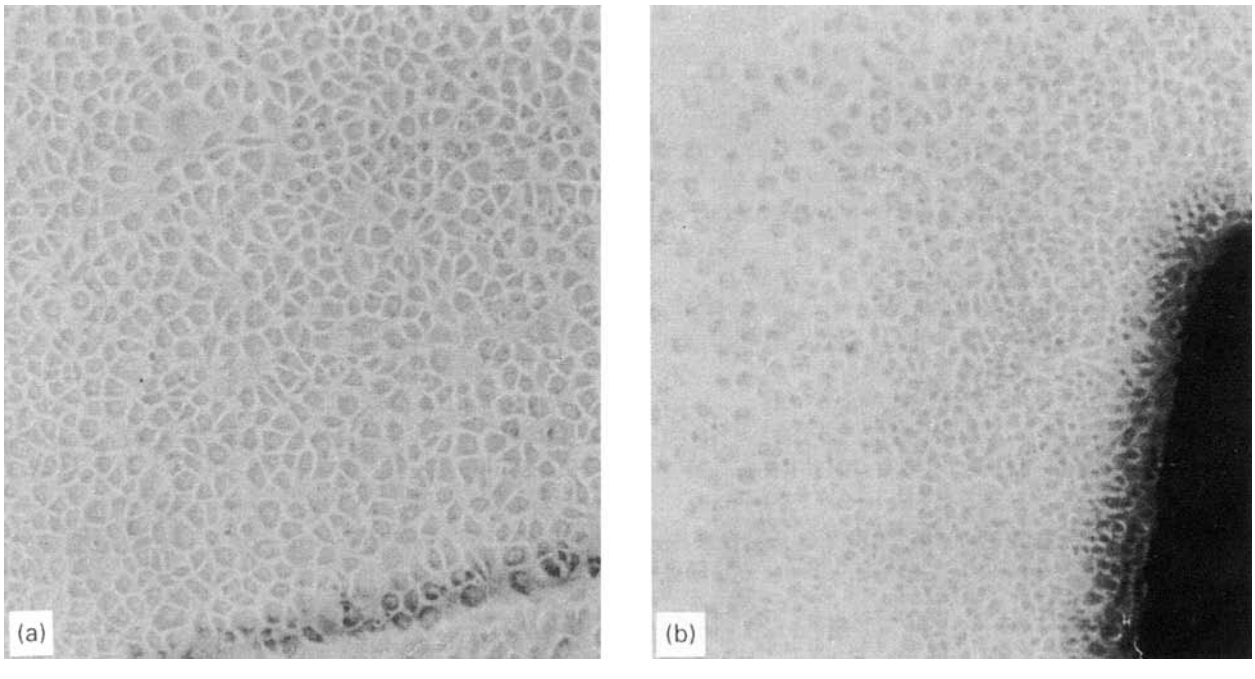

Figure 4 Morphology of cells: (a) on non-toxic control; and (b) in the vicinity of the hydroxyapatite plasma-sprayed coating.

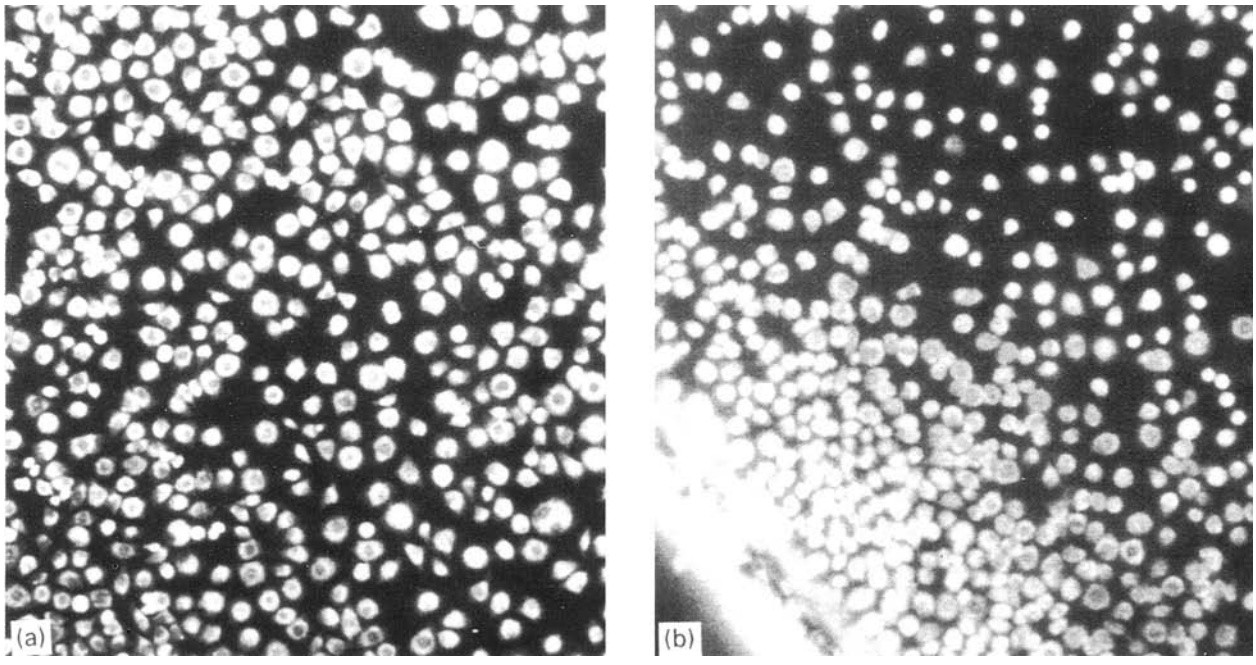

Figure 5 Fluorescence microscopy: (a) cells on non-toxic control, (b) cells on HAp plasma-sprayed coating.

of ethidium bromide and acridine orange of cells in the vicinity of the HAp and FAp samples, or in direct contact with these coatings, revealed that the fraction of cells with a red nucleus, which means a permeable, injured plasma membrane, was relatively small and comparable to that of the non-toxic control (Fig. 5).

Moreover, there is some evidence that there might be stimulation of cell growth by the HAp coatings. At the edges of these samples more cells were found than at the edges of the FAp samples. Thus, these results suggest that HAp would stimulate cell growth and FAp coatings would not.

4. Discussion and conclusions

Assuming that the released degradation products are only calcium-, phosphorus- and fluorine ions, some attempts to explain the stimulation in the cell cultures are presented.

In cell physiology calcium has the primary function of signal transduction. Working principles are transmission in cells and interaction with the environment. The condition for the latter is a high Ca^{2+} concentration gradient across the cell membrane. Dissolved calcium from the coating supports the establishment of high gradient and thus increases cell activity [7].

Phosphorus, however, is necessary for energy production and supply to the cell. It serves as a main coreactant in metabolic processes such as glycolysis and the citric acid cycle. Another function is to process the energy booster adenosine triphosphate. Therefore a high availability of phosphorous supports cell metabolism [7].

The reason for cell growth impairment in the presence of fluorine may be found in one of the following effects or their combination. It is possible that the pH factor of the aqueous nutritive solution changes as a result of reactions between dissolved fluorine ions and some free hydrogen ions. This change has an indirect influence on the cell metabolim as a result of an impaired enzyme activity. Additionally, the reaction product, named hydrofluoric acid has a high toxic effect [8].

Furthermore, the surplus of negative change produced by charged fluorine ions may shift the membrane potential of the cell and thus obstruct the exchanges with the environment [7]. This potential could remain constant if there are only double positive loaded calcium ions and triple negative loaded phosphorus ions in a proportion of 1.67:1 in the surrounding area.

In conclusion, varying the crystallinities of HAp did not lead to any problems. On the contrary, coatings with a higher dissolution rate support the growth of bony tissue because of the dissolved calcium and phosphorus.

Applying plasma-sprayed fluorapatite requires high coating stability, both mechanically and chemically, to prevent the negative effect of the fluoride ions in solution.

Acknowledgements

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