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Biocompatibility screening of silane-treated hydroxyapatite powders, for use as filler in resorbable composites

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This study reports *in vitro* biocompatibility screening of different kinds of silane-treated hydroxyapatite (HA) powders, for use as modified filler in resorbable composite. The silane coupling agents investigated were all methoxysilanes, with either vinyl (VS), or methacryloxy (MPS), or primary amine (AMMO), or secondary amine (TRIAMO), or diamine (DAS) functionality. Evaluation of cell adhesion on the different silanized HA surfaces, indirect agar-overlay test and direct contact test on extracts showed the acute toxicity of all five free silane agents, the innocuity of strongly adsorbed silane molecules on HA surfaces and the dose-dependent toxicity of leachable silanol molecules.

1. Introduction

In the research to develop biodegradable composites with good initial mechanical properties and resistance to water, hydroxyapatite powder has been used in many studies as a biocompatible and biodegradable filler [1–5]. Silane treatment of particles in order to optimize their performance as filler in composites has frequently been performed in composite technology, as for example for dental composites [6–9].

Prior to biocompatibility evaluation, the physico-chemical properties of the silanized mineral phase have been investigated in regard to physical factors (size, distribution, shape, etc.) as well as chemical factors (surface chemical composition, coating strength, dissolution, etc.) [10]. Silanized HA powder has been developed as part of resorbable composite materials, elaborated for the fixation of bone fractures. In this case of biodegradable composites, biocompatibility consideration should concern the HA bulk filler as well as the silane coating on these particles. Implant materials and their degradation products must be non-toxic at the systemic and cellular level. As far as we know, such biocompatibility studies on silanized hydroxyapatite has not been extensively reported [7].

Therefore, we carried out a cytotoxicity study, investigating the behaviour of cells in contact with the silane-treated HA filler. Different silane coupling agents were screened as coatings on mineral substrates, in order to provide more knowledge about the biological performances of different kinds of silane-treated HA filler.

As a first parameter of biocompatibility the ability of the cells to attach to the material surface during the early period of cell/material interaction was used. Then the different silane-treated powders were screened *in vitro* for cytotoxic effects on cultured fibroblast cells. This was performed by indirect agar overlay tests using either the silanized powders themselves or their extracts in saline. The composition of the extracts was analysed by high pressure liquid chromatography (HPLC) and the stability of the saline coatings to extraction was tested by X-ray photoelectron spectroscopy (XPS).

Considering that the implant material was degradable, a direct contact test using extracts was also performed to assess with greater sensitivity the toxic effects of the chemical entities being released from the treated powders.

2. Materials and methods

2.1. Materials

This biocompatibility screening concerned hydroxyapatite (HA) powders with a mean grain size of 20 μm (CAM Implants B.V., Leiden, NL), which were surface treated with different silane coupling agents (Aldrich Chemie, Belgium):

VS	Vinyltrimethoxysilane $CH_2=CH-Si(OCH_3)_3$
MPS	3-(trimethoxysilyl)propyl methacrylate $CH_2=C(CH_3)-C(O)O(CH_2)_3-Si(OCH_3)_3$
DAS	N-(3-trimethoxysilylpropyl) ethylenediamine $NH_2-CH_2-CH_2-NH-(CH_2)_3-Si(OCH_3)_3$
AMMO	3-aminopropyltrimethoxysilane $NH_2-(CH_2)_3-Si(OCH_3)_3$

TRIAMO N-methyl-3-aminopropyltrimethoxysilane
CH_3–NH–$(CH_2)_3$–Si$(OCH_3)_3$

The silane coating procedure was performed as previously described [10], depending on the silane functional groups. In short, low concentrations of silane (0.2–1% w/w) in aqueous alcohol solution (ethanol 90%) were used in order to retain the coupling agent as monomer or dimer to optimize its activity [9]. The hydrolysis of the methoxygroups was catalysed with acid in the case of VS and MPS, while the aminosilanes were auto-catalytic. HA particles (10 g) were silanized by stirring them in solution. After half an hour, the pH was increased in the case of VS and MPS. High pH encourages condensation and formation of siloxanols [9], which strengthen the coating. After 1 h reaction time, the powder was filtered and dried at room temperature (24 h) before being heat treated (100/120 °C; 2 h). Treated fillers were rinsed with cold solvent, dried and sieved (<45 μm).

For the cell adhesion test, HA tablets were used instead of powder. The tablets were made from pressed blocks (10 MPa) of sintered HA (1180 °C, 12 h) (CAM Implants B.V., Leiden, NL), which were cut with a diamond saw to obtain 2 mm thick squares. The tablets were then polished with sandpaper (800, 1200, 4000) and washed in ethanol solution in an ultrasonic bath. The surface roughness of the HA tablets was determined before treatment with a "Hommel Tester" equal to $R_a = 0.03$ μm ($R_z = 0.30$ μm). Just as the powders, HA tablets were silane treated from aqueous alcohol solutions. Only two representative silanes were used for this test: DAS, to check the influence of amino groups on the behaviour of the cells and VS to check the influence of the vinyl groups and also of hydrophobicity. The different chemical compositions of the silanized surfaces were studied by X-ray photoelectron spectroscopy (XPS; SSI 100, S-probe, Fisons, VG-group). The surface energies of the silanized tablets were obtained according to the sessile drop method with an optic goniometer (Kyowa). The measurements were made at room temperature with distilled water, Dimethylformamide (DMF) and formamide, on dessicated tablets (1 h at 40 °C prior to the experiment). Calculations of the surface energy and the polar and non-polar components were made according to Young/Dupré–Owens/Wendt. The surfaces of the silanized tablets were examined by scanning electron microscopy (Philips SE 525 M) to assess the differences in roughness and topography, which could possibly interfere with the biological response.

2.2. Extract liquid preparation

The extract liquid of the silanized powders was prepared according to the international standard (ISO 10993–5, "Biological evaluation of medical devices – Part 5: tests for cytotoxicity: *in vitro* methods", 1992). Culture medium without serum, i.e. DMEM, Dulbecco's Modified Eagles Medium (Gibco) with pH 7.2, was chosen as extraction medium for the direct contact test, while PBS (phosphate buffer solution) was used for the indirect contact test. The extraction was carried out in sterile polystyrene vials, under sterile conditions and soft constraints (37 °C, 120 h, without stirring). As specified in the norm ISO for powder samples, 0.2 g/ml was used as extraction ratio. After 5 days, the pH of the extracts were measured and adjusted to 7.2 with HCl solution. The extraction liquids were filtered through a membrane filter (<0.2 μm) and in the case of DMEM extracts, 5% foetal calf serum (FCS) (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Boehringer) were added to obtain a complete nutritive medium, which will be referred in the following as "test medium".

The calcium concentration of the different extracts was measured by atomic absorption spectrometry (AAS; SpectrAA-300, Varian Techtron, Australia) and their osmolarity was determined by freezing point depression (Roebling Osmometer, Berlin, Germany).

The content of hydrolysed silane molecules in the so-called "test media" was estimated by referring to the measurement of aminosilanols in PBS extracts by high pressure liquid chromatography (HPLC) (GBC Scientific Equipment Pty Ltd, Australia: LC1150 HPLC pump, LC1200 UV/VIS detector, LC1440 solvent conditioning system and LC1610 autosampler). Aminosilanes (AMMO, TRIAMO, DAS) were detected with a UV detector after a derivatization procedure with FMOC-CL reagent (9-fluorenylmethylchloroformate) [11, 12]. In short, 450 μl aminosaline standard solution in PBS (hydrolysed) or PBS-extract of aminosilane-treated HA powder were mixed with 50 μl borate buffer (1 M, pH 6). 500 μl FMOC-Cl reagent (5 mg FMOC-Cl in 50 ml acetonitril) were added. Derivatization time was 10 min; 20 μl of the resulting solution was injected in the chromatograph. A Zorbax RP-C8 Stable Bond column (150 × 4.6 mm) and guard column (10 × 4 mm) with the same material (Rockland Technologies, USA), both packed with 5 μm spherical particles, were used. The UV wavelength was set at 264 nm. Elution was performed by a gradient: solvent A was acetonitril, solvent B was 1.3% v/v acetic acid in water. The solvent composition changed according to the following scheme:

Time (min)	A (%)	Time (min)	A (%)
0–2.5	40	8–10	55–70
2.5–6	40–47 5	10–15	70
6–8	47.5–55	15–20	70–40

The flow-rate was set at 1.25 ml/min.

Peak integration and calculation were performed using PL Caliber™ LC/GC Software (Version 5.0, based on Microsoft Windows 3.1, from Polymer Laboratories, UK)

2.3. Cells and cell lines

L 929 clone mice fibroblast cells were chosen for the adhesion test because of their well-defined

morphology and reproducible phenotypic expression, while more sensitive primary human cells were used for the other tests. Human fibroblasts were isolated from the dermal part of a human foreskin biopsy (obtained after dispase (Boehringer) treatment). After Trypsin incubation (0.3% in NaCl, pH 7.6; 60 min at 4 °C and subsequently 30 min at 37 °C), the detached fibroblasts were resuspended and inoculated in a culture disc, using Dulbecco's Modified Essential Medium (Gibco) supplemented with 5% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml), designed as a "standard medium". The medium was refreshed three times a week. After two weeks confluent cultures, the cells were trypsinized and subcultured using a seeding density of 10^5 cells per 35 mm well. The fibroblasts were cultured at 37 °C and in a humidified atmosphere of 90% air/10% CO_2. The sixth and seventh cell passages were used for the experiments.

2.4. Test of cell attachment

Tablets (1 cm^2) were placed into six-well tissue culture plates. A suspension of L 929 cells in a droplet of 50 μl (10 000 cells/cm^2) was carefully added on top of the tablets. Ten minutes were allowed for the cells to attach before 3 ml of complete medium were added. As control, Thermanox films were used. Non-treated, VS-treated and DAS-treated hydroxyapatite tablets were tested. For each experiment, three tablets were used. After 24 h incubation, the cells on two samples were washed with excess of EDTA solution and then trypsinized. The retrieved cell suspension was coloured with trypan blue and the concentration of live cells was determined using a Malassey cell. On the other sample, cells were fixed with 1.5% Glutar-dialdehyde in 0.14 M cacodylate buffer (pH 7.2). and washed with cacodylate buffer before being dehydrated in an ethanol series. They were critical-point dried (Balzers CPD 010) and gold sputtered. The cell morphology was observed by scanning electron spectroscopy (Jeol JSM 6300) and cell attachment as well as cell density was estimated.

2.5. Indirect contact test

Silanized HA powders, free silane reagents, and the PBS extracts (Section 2.2) were screened for cytotoxicity by standard tissue culture agar overlay assay [13, 14]. For the experiments, human skin fibroblasts of passage 6 or 7 were used. After subculturing, the formation of a cell monolayer occurred in 5 days. As the culture was confluent, the culture medium was replaced by 2 ml Minimum Essential Medium containing 5% FCS, 3% AGAR (Bacto-agar, Difco Lab, Michigan), previously boiled in distillated water, penicillin (100 U/ml) and streptomycin (100 μg/ml).

0.2 g of sterilized powder was spread over an area of about 1 cm^2 of a solidified AGAR layer, while 50 μl of the test liquids (extracts or saline solutions) were dropped on filter paper (type HVLP 01300, pore size 0.45 μm, Millipore Corporation, Bedford, MA), placed on top of the AGAR layer.

TABLE I Evaluation of cytotoxicity for agar overlay test [15]

Sample	Reaction	Description of changes
Treated powders	−: not toxic	The cell monolayer is uniformly red in and around the position of the sample
Extracts	+ mild toxic	More than 20% of cells are round, loosely attached, occasionally lysed
Free silane agents	++. marked toxic	More than 50% of cells are round and devoid of coloured intracytoplasmic granules. Extensive cytolysis and empty areas between cells are noted

As positive control, 0.45% phenol solution was used, while the culture "standard medium" was chosen as negative control.

Samples and controls were tested in triplicate. After 24 h incubation, samples were removed and the cells were stained with 0.01% Neutral Red (Sigma) in PBS for 2 h at 37 °C. The cells were evaluated using an inverted microscope, and cytotoxicity was apparent by the lack of affected cells to take up Neutral Red.

To quantify the toxic response, we used the evaluation protocol of Oscar Sudilovsky [15] and described in Table 1.

2.6. Direct contact test

Primary human fibroblast cells were plated at a density of 2×10^4 cells per 1.5 cm well (24-well tissue culture plate, Greiner) and at 1×10^3 cells per 6 mm well (96-well tissue culture plate, Costar). The fibroblasts were cultured in standard conditions (Section 2.3). On day 3, the "standard medium" was replaced by the "test media". Three 1.5 cm wells per test medium were evaluated on day 4, 7, 10 and 14 for cell proliferation, three 1.5 cm wells for viability and six 6 mm wells for MTT assay (see below).

As negative control, the "standard medium" was used and as positive control, a solution of DAS coupling agent in standard medium 0.45% (v/v).

2.6.1. Cell proliferation

At the different evaluation days, the cells were harvested by trypsinization and counted using a Coulter counter (Coulter Electronics LTD Dunstable BEDS, England). After cell seeding, the number of cells was determined in the "standard medium", to obtain a reference curve. The cell numbers were plotted against the culture time.

2.6.2. Cell viability

At the observation times, the cells were washed with phosphate buffered saline (PBS) and incubated with 0.001% Neutral Red (Sigma) in PBS. After 30 min, the vital stain was removed and the cells were examined under an inverted microscope.

TABLE II XPS results on silanized HA tablets

Sample	Ca (%)	P (%)	C (%)	O (%)	Si (%)	N (%)
HA	14.68	9.16	26.98	44.64	–	2.31[a]
VS	3.35	4.75	31.21	44.32	9.92	3.37[a]
DAS	2 82	2.20	51.51	26.31	7.70	9.45

[a] Contamination

TABLE III Contact angle measurements on silanized HA tablets

Sample	Wettability (°)			E (mJ m^{-2})		
	Water	DMF	Formamide	Es	Dispersive	Polar
HA tablet	54	28	52	45.4	14.9	30.5
VS treated tablet	78	22	60	34.2	26.6	7.6
DAS treated tablet	50	26	50	48.6	14.2	34.3

2.6.3. *MTT assay Invittox Protocol, MTT assay, N° 17, ISSN 0960-2194, 1990*

At the different evaluation times, 10 µl of MTT solution (Sigma) were added to each well and the plates were incubated for 4 h at 37 °C. Then the MTT solution added to the medium was removed and replaced with 100 µl DMSO (dimethylsulfoxide) (J.T Baker Chemicals B.V., Deventer, NL), to dissolve the formazan. The plates were agitated for 5 min and "read" at 550 nm on a scanning multiwell spectrophotometer (ELISA, Bio-rad model 2550, Japan) to record the optical density of each well. The percentage of cell survival was expressed as:

$$\frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \times 100\%$$

All results were obtained from the mean of six determinants.

3. Results

3.1. Surface characterization of silanized tablets

Before cell inoculation, the silanized HA surfaces observed by means of SEM showed similarities in topography. The roughness was, however, slightly increased in the case of VS surface treatment.

XPS spectra gave the chemical analysis of the upper surface of the test samples (Table II). The large decrease in Ca and P atom percentages at the surface of silanized samples indicated the presence of a coating on HA tablets. The presence of Si and N elements was indicative of the chemical nature of the coating, although contamination with iron, magnesium and nitrogen (possibly due to the grinding and polishing processes) disturbed quantitative interpretation. However, the thickness of the coating could be roughly evaluated to be 5–10 monolayers, if we consider the thickness of a silane monolayer to be equal to 1 nm [9], and the scan depth in XPS to be 10 nm.

TABLE IV Osmolarities of the different test media

Test media	Osmolarity (mosm)
Standard medium	326
HA extract	335
VS treated HA extract	332
MPS treated HA extract	334
AMMO treated HA extract	332
DAS treated HA extract	332
Silane solution	393

TABLE V Calcium content in the different test media

Test media	Ca content (ppm)
Standard medium	66.4
HA extract	37.6
VS treated HA extract	37.5
MPS treated HA extract	34.8
AMMO treated HA extract	37.3
DAS treated HA extract	35.7
Silane solution	55.3

TABLE VI Concentration of hydrolysed silane molecules found by HPLC in PBS extracts of aminosilane-coated HA powders

Sample	Hydrolysed silane concentration (µg/ml)	Quantity of coating released
HA	0	0
DAS	52	1/3 monolayer
AMMO	41.5	1/2 monolayer
TRIAMO	6.5	1/18 monolayer

The results of the contact angle measurements are gathered in Table III. HA presented a high surface energy with a strong polar component. Vinylsilane treatment lowered the surface energy of HA tablets, especially the polar component, which showed clearly the hydrophobicity of the treated surface. Treatment with diaminosilane did not significantly change the wettability characteristics of the HA tablets.

3.2. Extract liquid analysis

All the "test media" were found alkalized, with a pH around 8.5, before being adjusted to 7.2 with HCl solution.

The osmolarities of the extracts of powders showed tolerable values [16] around 335 mosm/l (Table IV) while the osmolarity of the standard medium was equal to 326 mosm/l.

The "test media" contained less calcium than the "standard medium", as measured by atomic absorption spectrometry (Table V).

The HPLC analysis of the PBS extracts of AMMO, DAS, TRIAMO coated powder revealed the presence of hydrolysed aminosilane molecules in low concentration (Table VI) but with good accuracy. In fact, good calibration lines could be obtained from

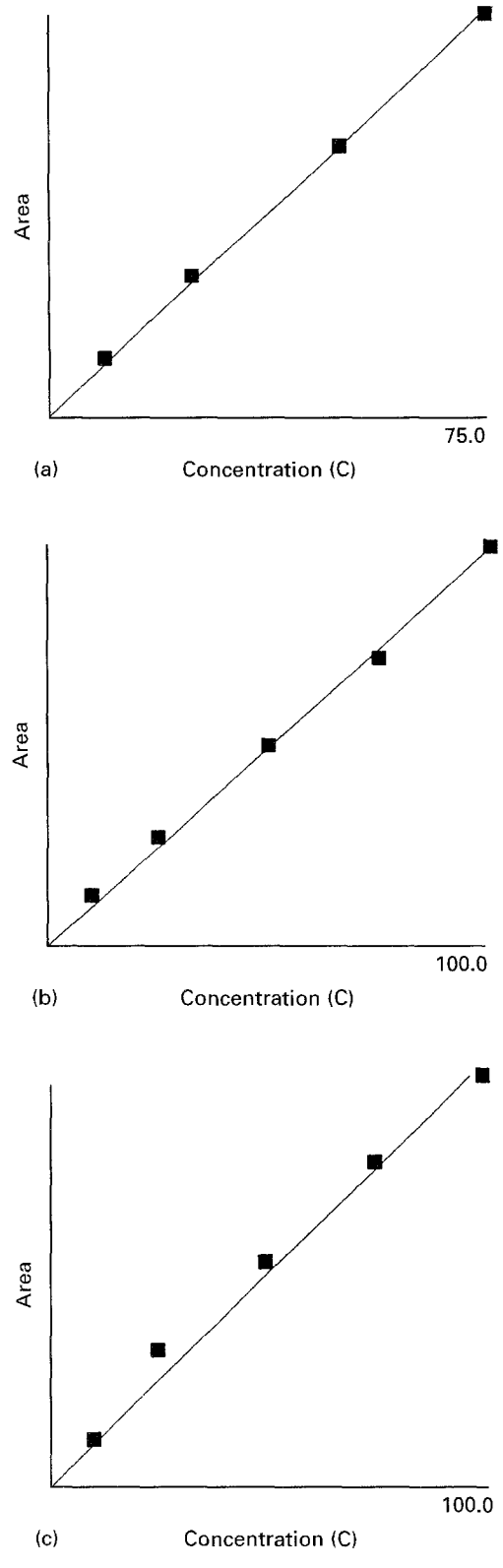


Figure 1 HPLC calibration lines for standard solutions of the three aminosilanes in PBS. (a) AMMO, r = 0 9991, (b) DAS, r = 0.9970: (c) TRIAMO, r = 0 9967.

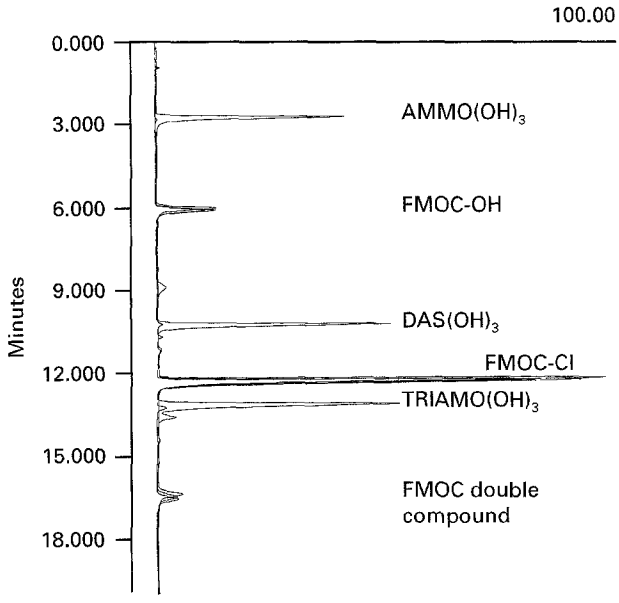


Figure 2 Superimposed chromatograms of standard solutions of the three hydrolysed aminosilanes in PBS after derivatization with FMOC.

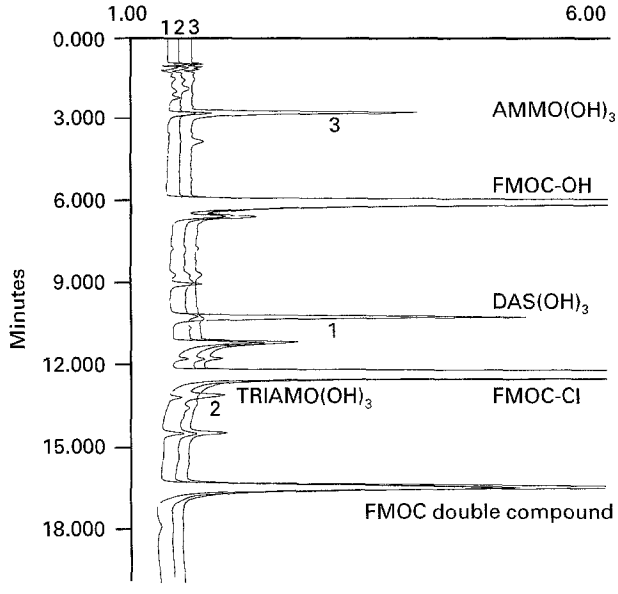


Figure 3 Superimposed chromatograms of PBS extracts of aminosilane-coated HA powder after derivatization with FMOC: (1) PBS extract of DAS coated HA powder; (2) PBS extract of TRIAMO coated HA powder; (3) PBS extract of AMMO coated HA powder.

AMMO, DAS, and TRIAMO in the hydrolysed state (Fig. 1). In all cases one extra peak in the chromatogram, besides the peaks of FMOC-Cl (12.2–12.3 min), FMOC–OH (6.0–6.1 min) and a FMOC double molecule (anhydride?) (16.3–16.5 min), could be observed (Figs. 2 and 3). The retention times for FMOC–AMMO(OH)$_3$, FMOC–DAS(OH)$_3$ and FMOC–TRIAMO(OH)$_3$ were 2.7–2.8 min, 10.3–10.4 min, and 13.1 min, respectively. No peaks, except for the FMOC reagent peaks, were seen in the analysis of a PBS extract of uncoated HA. Considering its retention time, we could assume that in the case of DAS, only the primary amino-group was derivatized. Under the described circumstances the detection limit for the three hydrolysed amino-silanes was around 100 ng/ml. With the following data: 3×10^{-6} moles silane were necessary to achieve a monolayer of 1 g of HA powder (specific surface: 0.935 m/g) [9], the molecular weight of the aminosilane and the extraction ratio (0.2 g powder/ml), the coating release corresponding to the silanol concentration could be determined (Table VI).

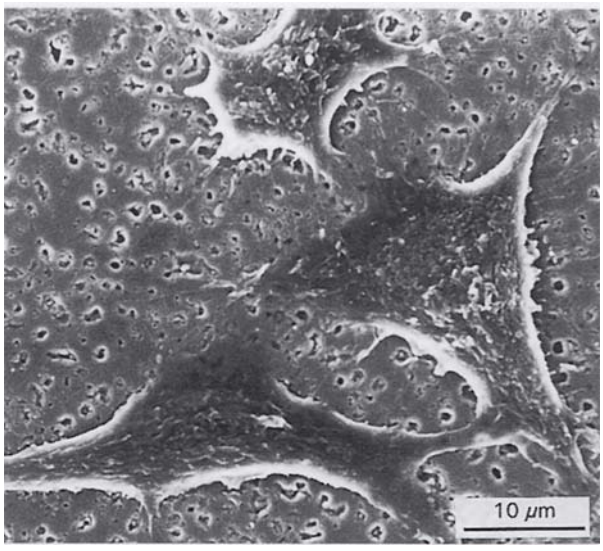


Figure 4 SEM picture: cell attachment on DAS-treated HA tablet.

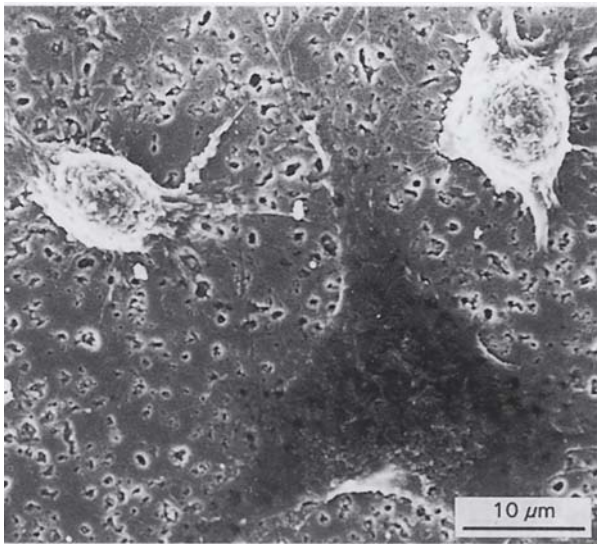


Figure 5 SEM picture: cell attachment on VS-treated HA tablet

Concerning VS and MPS coated materials, the extracts could not be analysed by HPLC with either a UV detector or a refractive index detector. The expected low quantity of released silanols and also the low solubility of these compounds prevented meaningful analysis.

3.3. Cell attachment test

After 24 h of L 929 cell spreading, the attachment pattern was found identical for non-treated HA surface and diamine silane (DAS) coated HA (Fig. 4). Cells adhered to many filopodias and were widely spread on the surface. A mild adverse effect was observed in the case of treatment with vinylsilane coupling agent (VS). Even if some cells could attach and spread, less were seen in intimate contact with the substrate, most were rounded off (Fig. 5). To confirm this adverse reaction, the density of cells present on the VS sample was found to be less than on the HA surface (Table VII), while no difference was noted in the case of aminosilane-coated tablets.

TABLE VII Number of cells attached to silanized HA tablets after 24 h culture[a] (cell seeding: 10^4 cells/tablet)

Sample	Cell concentration ($\times 10^4$ cells/ml)
Thermanox	18.1 ± 5.1
HA tablet	18.0 ± 6.9
VS treated tablet	12.6 ± 5.3
DAS treated tablet	19.7 ± 4.6

[a]Results obtained from triplicate measurements on two samples.

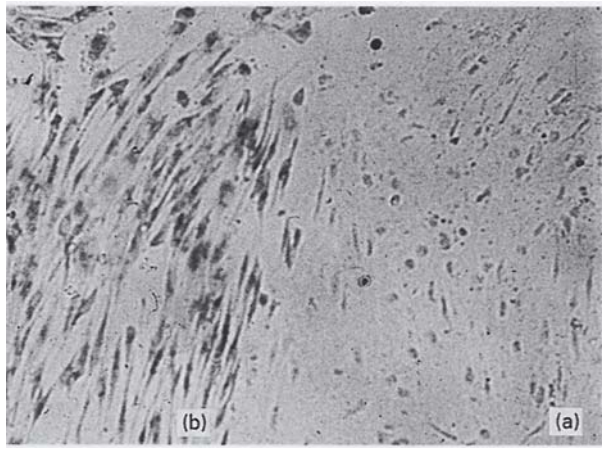


Figure 6 Cytotoxic response to DAS free silane agent (X200): (a) cell death under the sample; (b) cell affect at the sample periphery

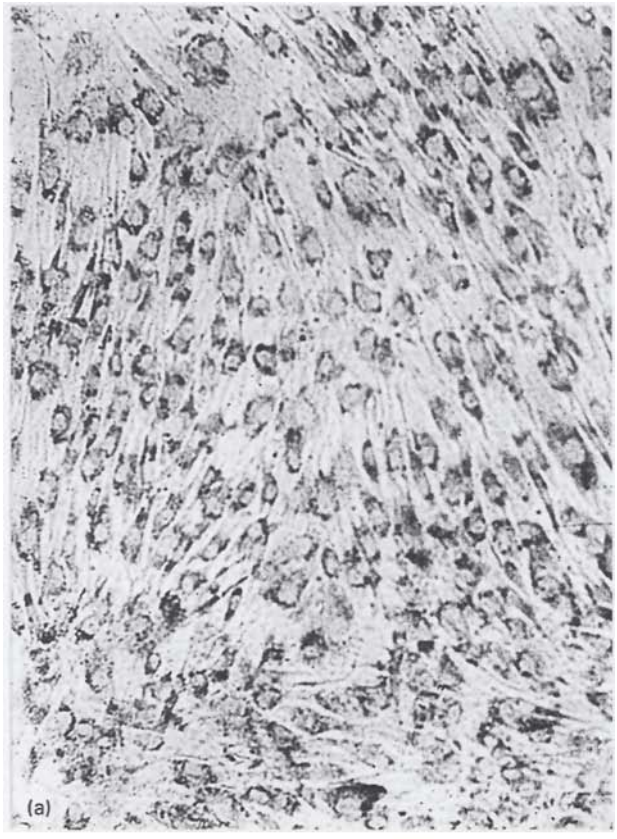
3.4. Indirect contact test

Powders were rated not acutely cytotoxic (−) as determined by the agar overlay test (Table I). The cell monolayer showed no damage after 24 h indirect exposure to powder samples. All free silane reagents showed significant cytotoxicity (++). Application of the test liquids led to cell lysis in a zone greater than the position of the sample (Fig. 6). The toxic reaction was less in the case of aminosilanes (AMMO, TRIAMO, DAS) than in the case of VS or MPS coupling agent solutions.

The extracts affected the density of the cell monolayer under the Agar diffusive barrier, indicating a mild toxic reaction (+). Some morphological changes, such as rounding or floating of the cells, was also noted (Fig. 7). The toxic response differed slightly among the different extracts, correlated with the amount of hydrolysed aminosilane molecules which could be detected in the extraction liquid (Table VI).

3.5. Direct contact test

Each "test medium" allowed proliferation of the fibroblast cells. Moreover, the cell growth tended to be enhanced when the cells were cultured in the "test medium" (Fig. 8). This result was supported by a strong mitochondrial activity indicated by MTT assay (Table VIII). The cells cultured in test media showed a slightly different morphology to those cultured with standard medium. They were sometimes larger, strongly stained and with a rounded appearance.



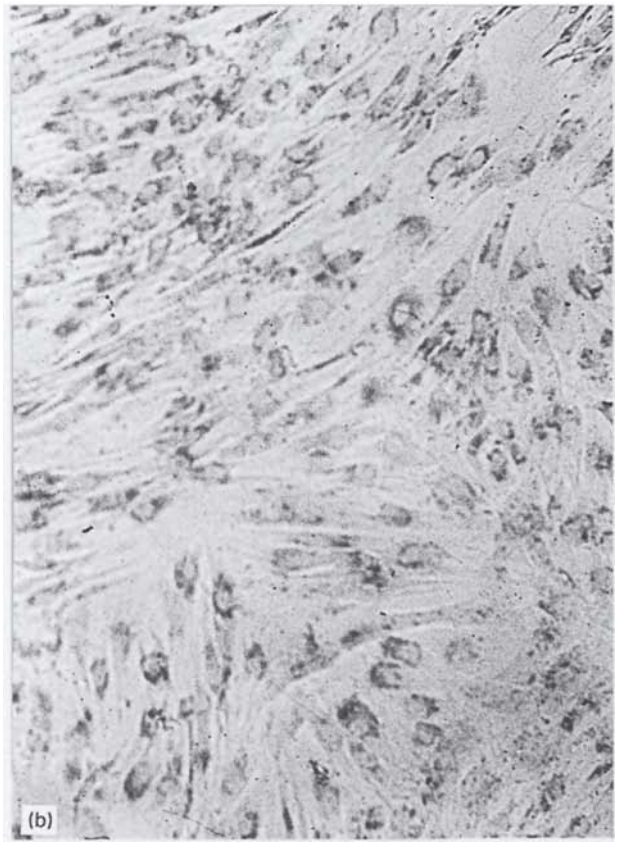


Figure 7 Mild toxic response to the extract of VS-treated HA powder: decrease of cell density (X 200): (a) control; (b) VS extract.

4. Discussion

To evaluate the cytotoxicity of a material, it is necessary to use several test methods to assess the effects of the multiple factors involved [17].

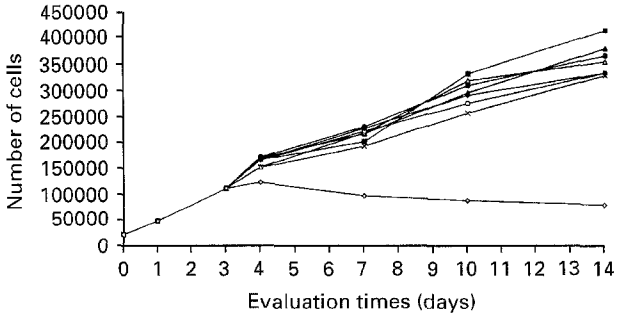


Figure 8 Cell proliferation curve. × medium. ▲ HA; ● MPS; ■ VS; △ AMMO. ○ DAS; ◆ TRIAMO; ◇ positive control.

TABLE VIII Results of the MTT assay: percentage of cell survival

	HA (%)	VS (%)	MPS (%)	AMMO (%)	TRIAMO (%)	DAS (%)	Silan (%)
Day 4	131	110	115.5	115	114.5	114	78
Day 7	119	120	140	142.5	131	143.5	39
Day 10	131	116	110 5	126.5	124	103	18.5
Day 14	95.5	88	101.5	89	89	84.5	9.5

Concerning the adhesion test, the investigated substrates showed different cell attachment patterns, which depended mainly on the chemical surface composition because the topographies of the different tablets were similar. The aminosilane (DAS) coating allowed the cells to attach on the hydroxyapatite surface but vinylsilane (VS) treated surfaces showed unfavourable conditions for cell attachment. The latter effect might be caused by toxicity but could also be imputed to high hydrophobicity since surface energy influences cell attachment [18].

In the agar overlay assay, the silanized fillers showed no toxicity but unbonded silane molecules caused cell death. Since HPLC characterized the releasing chemical entities in the extracts as hydrolyzed silane molecules, the degradation products of the silanized filler were expected to be toxic. Indeed, screened by indirect contact test, they seemed to affect the density of the cell layer as well as cell morphology, even through the diffusive agar barrier. However, the result of this test was not sufficiently clear to discriminate toxicity or not.

Higher sensitivity would be expected with the direct contact test based on extracts. Culture conditions were modified by using culture medium containing extracted products of the silane-coated powders. Calcium content in the "test media" was decreased in comparison with "standard medium". This could indicate partial dissolution of the ceramic powders in the extraction medium, followed by reprecipitation of a different calcium phosphate type on the filler surface. Calcium phosphate deposit of higher Ca/P ratio than hydroxyapatite (as, for example, carbonated apatite) was probably the reason for the change in ion homeostasis of the culture conditions. The decrease of calcium content in the culture medium might influence cell viability by weakening the cell membrane. In spite

of this calcium deficiency, an unexpected mitochondrial stimulation was observed by direct contact test with the extracts of silane-treated powders. However, no significant difference was determined between cell proliferations obtained with test media and those found with standard medium. Nevertheless, the silane extracts changed the cell morphology. Thus, at low concentration, hydrolysed silane molecules modified the cell behaviour but did not cause lethality. On the other hand, concentrated silane solution, chosen as positive control, strongly inhibited cell growth. Thus, the silanols must be considered as offending agents, able to modify cell morphology, but causing toxic reaction only at higher concentrations. This limit of toxicity has to be determined *in vivo* in the future.

Large amounts of degradation products might have a negative effect on the surrounding tissue but we have to consider that in the case of silane-treated HA filler, the maximum release of leachable products will be limited in any case by the thinness of the coating applied. In fact, as advised by Ishida [19], the optimum silane coating layer would correspond to a few firmly chemisorbed monolayers (2–3). Such a coating would be enough to achieve chemical bonding between the mineral fillers and the polymer matrix and would not be too thick to weaken the interface properties in a composite material. In this study, extraction at 37 °C over 5 days caused the leakage of only the equivalent of about one third of a silane monolayer, demonstrating the quality of adsorption of the silane coating on HA powder.

5. Conclusion

Although it was feared that silane coupling agents could bring about incompatibilities with living tissue, preliminary results of acute toxicity rated the biocompatibility of silanized powders as satisfactory in so far as the coating remained stable on HA filler. Hydrolysed, the silane molecules have adverse effects on cell morphology, and at high concentration cause cell damage.

In view of the known adverse biological activity of leachable products, it is essential to ensure high stability of the silane coating on the HA filler, i.e. to enhance the crosslinking of the siloxane barrier during the coating procedure.

If suitable for interaction with the polymer matrix, the use of aminosilanes has to be preferred over VS or MPS because of the advantage of hydrophilicity. Cell adhesion is enhanced on hydrophilic surfaces and the solubility of leachable products in aqueous fluid may favour their draining in the body.

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