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Biocompatibility of wear-resistant coatings in orthopaedic surgery *in vitro* testing with human fibroblast cell cultures

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The *in vitro* biocompatibility of six wear-resistant coatings that were devised as an alternative to polyethylene in resurfacing hip arthroplasties was assessed. The coatings were applied on glass-cover slips via the chemical vapour deposition (CVD) and physical vapour deposition (PVD) methods. Testing was performed in human fibroblast cultures and toxicity was evaluated by use of total cell surface quantitation, light and scanning electron microscopy. Multiple cracks were observed in the tungstenhydrogencarbide (WCH) and chromiumcarbide (CrC) coatings immediately after adding the growth medium. These surface alterations were not related to the presence of fibroblasts. Because of these surface changes, the general biocompatibility of WCH and CrC could not be assessed. Chromiumoxide (Cr_2O_3) caused severe alterations in cellular morphology followed by significant cytotoxicity. Alumina (Al_2O_3), titaniumnitride (TiN) and titaniumcarbide (TiC) coatings caused no adverse effects on cells in culture. The fibroblasts showed a rapid and logarithmically stable growth curve on these three materials. They merit further investigation as a wear-resistant coating for orthopaedic implants.

1. Introduction

Currently one of the main causes of hip arthroplasty failure is accumulation of polyethylene and metal particles in periprosthetic tissues [1–9]. A widespread foreign body reaction has been cited as a major contributor to osteolysis and loosening of hip arthroplasties. Mirror-finished pure titanium or Ti-6Al-4V sliding surfaces not subjected to surface treatment are particularly prone to extensive wear when articulating against polyethylene [10, 11]. Release of titanium and titanium–aluminium–vanadium alloy particles can cause an unfavourable biological response [12, 13]. Mechanical interaction during articulation leads to disruption of the non-shear-resistant oxide layer which consists mainly of TiO_2. *In-vitro* experiments showed that the polyethylene wear rate was observed to be about 100 times that encountered with stainless steel or cast Co–Cr–Mo alloy under the same conditions of testing. Excessive polyethylene wear is also generated when articulating surfaces of prosthetic components have a large diameter, as in resurfacing hip arthroplasties [14]. It has been suggested that future designs of resurfacing hip arthroplasties should be focused on wear-resistant materials.

The problem of polyethylene wear has raised new interest in metal–metal total hip arthroplasty. In studies of revision operations it was noted that in spite of the metal–metal combination, abrasion was slight, since staining of the peri-articular tissues was found only in one case [15]. The capsule appeared to be more or less normal. Another striking phenomenon during revisions of these all-metal hip arthroplasties was the very low wear and tear of the acetabular component despite a large 42-mm head. There was little or no reaction of the para-articular surrounding tissues. This was in contrast with the findings during revision of polyethylene–metal systems where, generally, severe foreign body reactions and bone lysis on femoral and acetabular side were observed. Another study showed that the polyethylene–metal combination showed 40 times higher wear in the polyethylene part [16]. Based on these remarkable long-term clinical results, metal/metal alloy has regained interest for hip arthroplasties.

The surface finish and sphericity of femoral heads are of paramount importance in minimizing the generation of wear particles in metal–metal as well as metal–polyethylene hip arthroplasties. Another approach in reduction of metal debris involves surface modification of the metallic surfaces with a wear-resistant coating. The requirements for coatings in load bearing and articulating implants are particularly exacting. Excellent adhesion between coating and substrate is of utmost importance, accurate dimensional tolerances have to be maintained thus favouring thin coatings (< 20 μm). For several years, wear-resistant ceramic coatings have been applied in machine bearings and for hardening tools, leading to increases in

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lifetime of between 300 and 800% [17–20]. Muller *et al.* [21] tested a ceramic coating in layers of 4–6 μm on chrome-cobalt heads and sockets. The harder titaniumcarbide (TiC) was preferred for the 37- and 42-mm heads and the softer titaniumnitride (TiN) for the socket (TiC–TiN) system. This combination has already been applied in clinical practice, but early and medium-term follow-up studies of these total hip arthroplasties have not been available until now. It is possible that these wear-resistant coatings will delaminate from the metallic substrate after a long-term period. Therefore it is essential to be informed about the general biocompatibility and biodegradation properties of these materials in addition to the already well-known favourable tribological properties.

We have screened six coatings on general biocompatibility in human fibroblast cell cultures.

2. Materials and methods

2.1. Coating characteristics (Table I)

Standard 16 × 16 mm glass cover-slips were covered with six different coatings (Philips PMF, Eindhoven, The Netherlands). Transparent glass cover-slips were used because in this application, light microscopic as well as scanning electron microscopic techniques could be applied.

The coatings concerned, titaniumcarbide (TiC), chromiumoxide (Cr_2O_3), chromiumcarbide (CrC) and alumina (Al_2O_3), were all applied by the chemical vapour deposition (CVD) method [19] (Table I). Titaniumnitride (TiN) and tungstenhydrogencarbide (WCH) were applied by the physical vapour deposition (PVD) method [17–20] (Table I).

The hardness of TiC (CVD) was 2800–3500 kg/mm. The hardness of TiN and WCH (PVD) were 1500–2500 kg/mm.

2.2. Cell culture technique

Stock cultures of human fibroblasts were initially stored in liquid nitrogen. The thawed cells were cultured with Dulbecco's Modified Eagles Medium (DMEM) and F12 in a 3:1 ratio to which hydrocortisone (0.4 M), isoproterenol (10^{-6} M), penicillin (100 U/ml), streptomycin (100 μg/ml) and 5% foetal calf serum had been added. After three days of culturing, Epidermal Growth Factor (10 ng/ml) was added to the growth medium. The cells were initially cultured in 10% CO_2 at 37 °C and harvested from 14-cm culture dishes by trypsinization. Cultures were grown in six wells on 35 mm diameter polystyrene plates in which the coated glass-cover slips had been inserted. The fibroblasts were cultured on the coatings for up to 14 days. All glass-cover slips were sterilized by gamma irradiation. On day 0, cells were seeded on these coated glass-cover slips at a density of 1.1×10^6 fibroblasts per well. To allow spreading and attachment, the cells were cultured for 3 days in this medium before adding growth factor. After 1, 4, 6, 10, and 14 days, the glass-cover slips were removed and the cells were fixed. For each experiment, controls (uncoated glass-cover slips) were processed in exactly the same way. Two aspects were considered: the amount of glass-cover slip surface that had been covered by fibroblasts; and the morphology of the fibroblasts with regard to cellular alterations and cytotoxicity. Cell growth was established with a VIDAS image analysis system. Furthermore, colony formation on the glass-cover slip was assessed. Ten evenly spaced fields of view were counted on each glass-cover slip. The total cell surface was determined in each field. For each condition, a triplicate procedure was performed making the total number of counts of one interval thirty for each condition.

TABLE I Coating characteristics

	CVD	PVD
Coating	TiC, Al_2O_3, CrC, Cr_2O_3	TiN, WCH
Thickness (μm)	5	5
Porosity	+ + +	+ +
Hardness (Vickers)	2000–3000	1000–1500
Stitch	+ +	+

2.3. Light microscopy (LM)

Cytotoxicity was analysed by light microscopic examination of the fixed and stained cover-slips using a phase contrast microscope. Fixation for light microscopy was performed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4, 4 °C) for at least 2 h. Staining for light microscopy was performed with toluidine blue.

2.4. Scanning electron microscopy (SEM)

Detailed information about cell surface morphology was obtained by scanning electron microscopy. For scanning electron microscopy, all specimens were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (370 mmol, pH 7.4) for 2 h at 4 °C. The fixed preparations were dehydrated in a graded ethanol series, critical point dried under carbon dioxide and covered, using a sputter coater with a layer of gold about 20 nm thick, before being examined with a Philips 525 M scanning electron microscope at an accelerating voltage of 15 kV and a tilting angle of about 20° relative to the electron beam. To investigate the influence of the culture medium on the surface properties of the coatings, glass-cover slips were treated in exactly the same way as described in the absence of fibroblasts. The amount of fibroblast covered surface was assessed for each coating on day 1, 4, 6, 10, and 14.

2.5. Statistics

Statistical analysis of the growth curves was performed using the multiple analysis of variance (MANOVA) of the logarithmic transformed covered surfaces

at a confidence level of 95%. This test revealed whether significant differences were present between the complete growth curves of the different materials. Statistical calculations were performed with the software package NCSS.

3. Results

First, the different CVD and PVD coatings and the control glass-cover slip in dry state were investigated by scanning electron microscopy (Table I). Multiple, small cracks were observed in the CrC and WCH layers immediately after adding the growth medium and fibroblasts (Fig. 1). After several days, spalling of the CrC coating occurred. The same phenomena were observed when the CrC- and WCH-coated glass-cover slips were placed in the growth medium but without addition of the fibroblast cell cultures. Consequently the surface changes of WCH and CrC were not related to the presence of fibroblasts. Because of these surface changes it was not possible to compare fibroblast proliferation on these two coatings with cell proliferation on the other four coatings and the uncoated glass-cover slip (control). Consequently, the CrC and WCH coating were excluded from further evaluation.

There were no differences or alterations observed in the surfaces of the other four coatings during the 14 days of exposure to the growth medium only or during 14 days of exposure to fibroblasts. The course of the growth curves on TiN, TiC, Al_2O_3 and Cr_2O_3 was significantly different in comparison to the growth curve on the control ($p < 0.005$). Plots of covered surface against time of exposure to the different coatings and controls are shown in Fig. 2. After 1, 4 and 6 days of culture, there were more colonies on the TiN coating than the Al_2O_3, TiC, Cr_2O_3 and controls. During the later phases of culturing, no differences between the number of colonies on the TiN and the TiC coatings were observed. After 14 days the number of cells on the Al_2O_3 coating was significantly less than those on both TiN and TiC coatings and control. From day 4 on, there were almost no cells left on the Cr_2O_3 coating, related to a massive cell lysis.

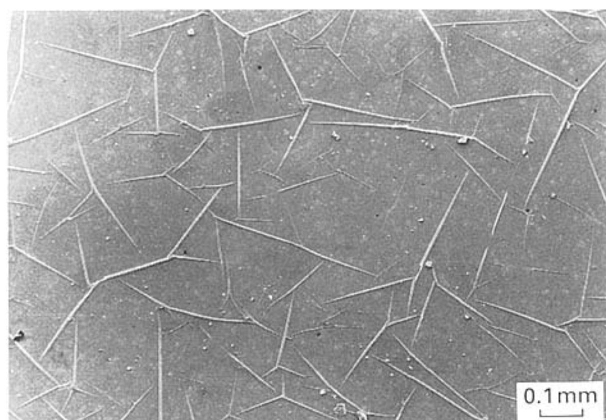


Figure 1 SEM of tungstenhydrogencarbide (WCH) coating after 1 day of culture. The surface shows multiple small cracks.

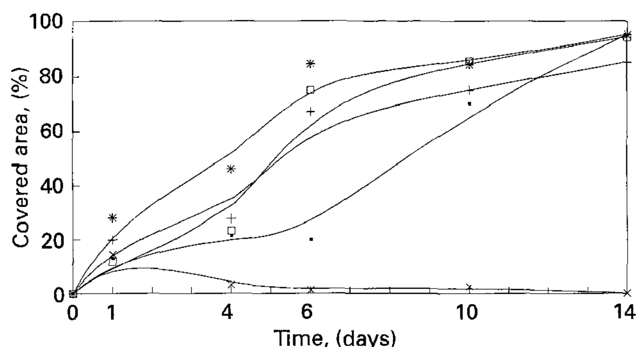


Figure 2 Growth curve of human fibroblasts on glass cover-slips covered with four different ceramic coatings and an uncovered glass cover-slip (control): □ control; + Al_2O_3; ∗ TiN; □ TiC; × Cr_2O_3.

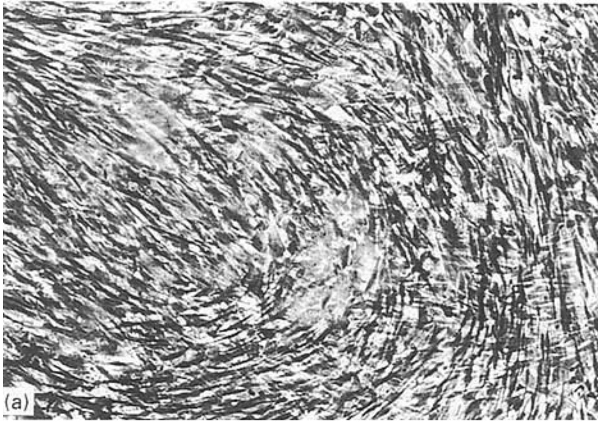
3.1. Light microscopy

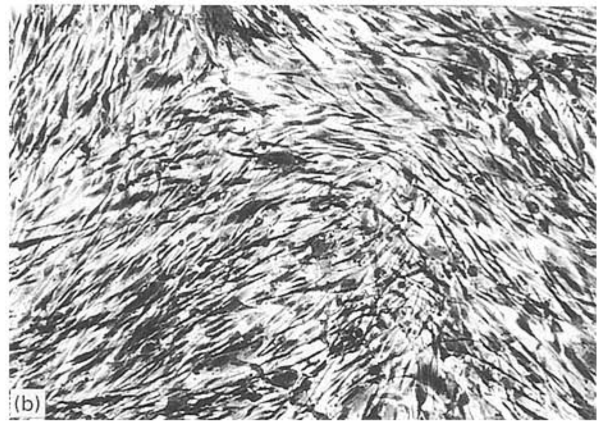
During the early phases of growth (after 1 and 2 days' incubation), the typical spindle shape of cultured fibroblasts was discernable. Cells grown on TiN, TiC and Al_2O_3 coatings were morphologically indistinguishable from those observed in control cultures. In general, the proliferation pattern of the cells cultured on these coatings was similar and comparable to the control cultures. The fibroblasts developed a stratification of cells combined with a parallel orientation of cells into tracks comparable with the control. The increase of covered surface was generated from small colonies eventually resulting in confluent cultures (Fig. 3a–d). Changes in cell morphology were observed after 2 days on the Cr_2O_3 coating. After 1 day, the cells showed morphological changes, such as withdrawal of cytoplasmatic processes, pyknosis and abnormal nuclear and cytoplasmatic staining. Cell necrosis became apparent as extensive cellular debris. During further exposure, cell growth was very limited and quickly followed by the death of the entire culture (Fig. 3e).

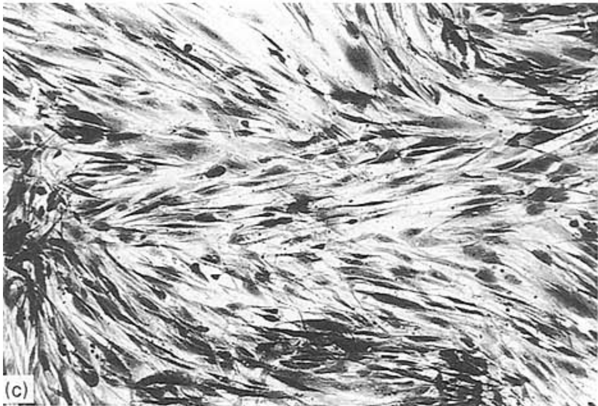
3.2. Scanning electron microscopy

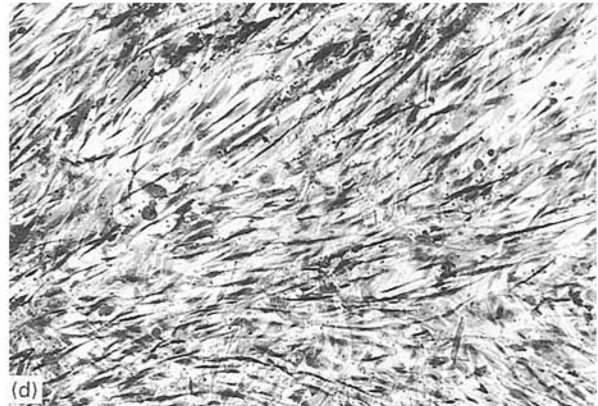
Except for the Cr_2O_3 coating, fibroblasts cultured on all other coatings showed a morphology that was similar to the fibroblasts which were cultured on the glass cover-slips that were used as a control (Fig. 4). These fibroblasts were rather flat, with a highly attenuated cytoplasm. The fibroblasts were attached with the tip ends of filopodia and microvilli to the glass-cover slips. The cells stretched their corners to extend slender processes and became triangular or polygonal. When the cells were polygonal, there were slender cell processes. The whole cell surface was covered with microvilli. Filopodia were found abundantly at the cell periphery. They varied in length from just touching the substratum to reaching another distant cell. Among pseudopodia there were those thinly spreading, those thickened like stairways and those that had retracted and formed at the bases small masses from which filopodia were growing. During the latter phases of culturing, several layers of fibroblasts became visible.

On the Cr_2O_3-coated glass-cover slip, the cell processes rapidly disappeared after 2 days. After that the









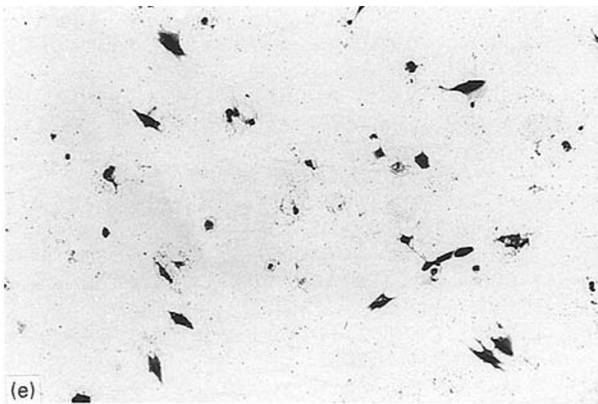


Figure 3 LM after 14 days of culturing (magnification ×200): (a) titaniumnitride; (b) titaniumcarbide; (c) aluminiumoxide (magnification ×400); (d) control; (e) chromiumoxide.

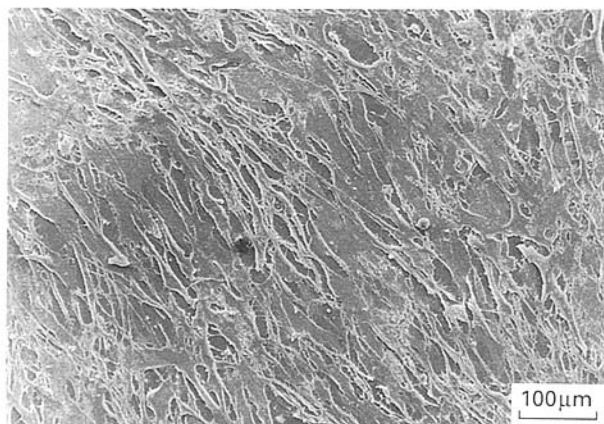


Figure 4 SEM of the fibroblasts on uncoated glass cover-slip (control) after 14 days.

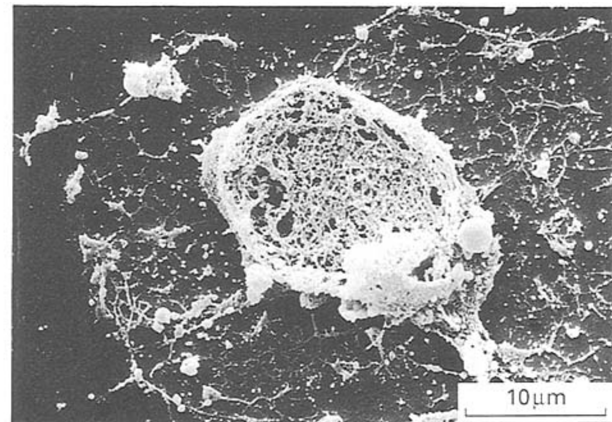


Figure 5 SEM of fibroblast on Cr_2O_3 after 6 days showing significant alterations of cellular morphology.

cell rounded up. In the latter phases there was only cell debris visible, in which the normal morphology, as was seen on the other, different coated glass-cover slips, had been totally lost (Fig. 5).

4. Discussion
Polyethylene is one of the weak links in the resurfacing hip arthroplasty. Excessive polyethylene wear causes foreign body reactions in the periprosthetic tissues

and resorption of bone. Recently it was suggested that future designs of these arthroplasties should be focused on the use of new generation wear-resistant material combinations. In this experiment, six wear-resistant coatings that might be applied in a metal-to-metal-bearing prosthesis have been tested on general *in vitro* biocompatibility. Fibroblasts were directly exposed to the materials. It is clear that *in vivo* assessment of the biocompatibility of implant materials by nature reflects the end result of a number of assaults on the tissue, such as surgical trauma, interface stress and micromovement, leachables from the implant, and surface physical phenomena. Therefore, all these factors must be taken into consideration in order to reach a reasonable conclusion on biocompatibility of new materials that are introduced. Many authors have reported several methods for assaying the biocompatibility of biomaterials [22–24]. These methods have some limitations, such as possible differences in the results depending on the cell type or assay method but they have the advantage of sensitivity, reproducability, speed, and adaptability for assaying.

In our experiments, human fibroblasts were selected for the screening tests. Our method concerned the method of direct contract to the concerning coatings. In the cytotoxicity test on these biomaterials, our results may be affected by both the chemical and physical properties of the materials.

A striking phenomenon was the multiple cracks in the surfaces of the WCH and CrC coatings directly after adding the growth medium. Chemical interactions of the medium with both WCH and CrC coatings could be responsible for this phenomenon. Probably, these layers were not chemically inert. TiN, TiC, and the Al_2O_3 coatings showed no differences from the control in cytotoxicity. This favourable biocompatibility could be a reflection of the well-known low solubility and high corrosion resistance. The growth curves of the different materials were different. This difference could not be attributed to a time-limited effect.

It is still uncertain whether the significant cytotoxic effect of Cr_2O_3, as determined by cell death and lack of colony formation, was related to the chemical composition of Cr_2O_3 or one of the physical properties. Presumably, the Cr_2O_3 coating exerted negative effects by dissolving in the culture medium to release metallic ions. It is well known that chromium ions can be responsible for undesirable cytotoxic reactions [25]. However in this experiment, ion release into the growth medium has not been assessed.

The methods used in our experiments are intended as a rapid evaluation of the coatings to be tested and it was shown by the results of this study that the Cr_2O_3, WCH and CrC coatings could be excluded from further experiments because of cytotoxic or mechanical reasons. Extrapolation to the *in vivo* situation is a problem. These *in vitro* experiments represent only one phase in studying biocompatibility. The TiN, TiC and Al_2O_3 coatings were classified as *in vitro* biocompatible and must enter a further phase of testing which requires *in vivo* observation. Surface modification of hip resurfacing metal components with TiN, TiC or Al_2O_3 in the last stage of manufacturing could possess an optimum combination of environmental integrity, biological response and favourable tribological properties. Future studies will be directed to application of these layers in a metal-on-metal device on tribological aspects [26], their behaviour as particulates in mouse peritoneal macrophage cell cultures and the biomaterial breakdown.

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